On the osmotic signal and osmosensing mechanism of an ABC transport system

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Voor mijn ouders

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On the osmotic signal and osmosensing mechanism of an ABC transport system

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Tiemen van der Heide

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Voorwoord

Eindelijk is het dan zover! Na tweeënhalf jaar ‘gisten’ en drie jaar ‘stress’ is het ook mij gelukt. De afgelopen jaren zijn voor mij een periode geweest waarin ik op meerdere vlakken veel heb geleerd. Als ‘kleine zelfstandige ondernemer’ kom je tijdens je onderzoek allerlei obstakels tegen die soms het uiterste van je vergen. Het is dan ook belangrijk om pieken en dalen in het onderzoek te nemen voor wat ze zijn, en zodoende je grenzen te verleggen.

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Lieve Esther,.....ik ben blij dat je er bent.

Tiemen

Contents

General introduction

I. General features of cell volume control

In bacteria, the intracellular concentrations of osmotically active solutes are very high, resulting in an outward pressure on the cytoplasmic membrane. To prevent the membrane from rupturing, bacteria have an external wall, termed the sacculus or exoskeleton. The resulting outward pressure that acts on the cell wall, often referred to as turgor (-pressure), arises from the osmotic imbalance between the intra- and extracellular compartments. Both Gram-negative and Gram-positive bacteria are surrounded by a cell wall consisting of peptidoglycan, which has to resist turgors ranging from 0.8 to 5 atm. and 15 to 25 atm., respectively (110).

In their natural habitat bacteria can be subjected to several types of stress, disturbing vital cellular processes (Fig. 1). When subjected to an osmotic upshift (an increase in the medium osmolality), the bacterium is faced with dehydration and a decrease in turgor. Maintenance of turgor above a certain critical level is essential as it provides the mechanical force for expansion of the cell. To restore a decrease in turgor, bacteria raise the intracellular osmolality via the accumulation of compatible solutes, which are compounds that can be synthesized or taken up to high cytoplasmic concentrations without disturbing vital cellular processes. To accumulate these solutes, bacteria deploy transport systems that are induced/activated upon osmotic upshift. In terms of the energy-coupling mechanism they can be of the type that bind and hydrolyze ATP to drive the translocation of substrate against the concentration gradient or transporters that drive the uptake of substrate by using the electro-chemical ion-gradients across the membrane (110).

When subjected to an osmotic downshift (a decrease in the medium osmolality) bacteria have to deal with a rapid influx of water, and thus a rapid elevation of turgor. In the ultimate case, too high a turgor endangers the integrity of the cell by rupturing the cytoplasmic membrane and the cell wall, resulting in cell death. To prevent cell lysis from happening, so-called mechanosensitive channels are activated, resulting in the expulsion of solutes down their concentration gradient.

Definitions (157)

Chaotropes are cosolvents that decrease water structure. Urea and other protein denaturants are chaotropes.

Chemosensors are molecules that detect specific ligands. Many chemosensors act by binding a specific ligand at a structure-specific receptor-site.

Compatible solutes are cytoplasmic cosolvents whose level can be modulated over a broad range without disrupting cellular functions.

Cosolvents are solutes that significantly affect the properties of water as a solvent, rendering the resulting solution nonideal.

Dehydration is water loss (desiccation is complete water removal).

Kosmotropes are cosolvents that increase water structure. Glycerol, glycine betaine, and other protein stabilizers are kosmotropes.

Osmolality is the osmotic pressure of a solution at a particular temperature, expressed as moles of solute per kilogram of solvent (osmol/kg or osmolal). Osmolality can be measured but not calculated.

Osmolarity is an approximation for osmolality, expressed as moles of solute per liter of solution (osmol/l or osmolar). Osmolarity is calculated as the sum of the concentrations of osmotically active solutes in a solution.

Osmoprotectants are compounds that stimulate bacterial growth in high-osmolality media.

An **osmoregulatory response** is a physiological process that mitigates passive adjustments in cell structure caused by changes in the extracellular osmolality.

Osmosensors are devices that detect changes in extracellular water activity (direct osmosensing) or resulting changes in cell structure or composition (indirect osmosensing).

Osmotolerance is the osmolality range for the media that support bacterial growth.

Osmotic downshift is a decrease (over time) in the osmolality of the extracellular environment.

Osmotic upshift is an increase (over time) in the osmolality of the extracellular environment.

Turgor (ΔP) is the hydrostatic pressure difference that balances the difference in internal and external osmolyte concentration. In equation:

$$\Delta P = (RT/V_w) \ln(a_o / a_i) \sim RT(c_i - c_o)$$

in which V_w is the partial molal volume of water, a is the water activity, c is the total osmolyte concentration, and the subscripts i and o refer inside and outside, respectively. Turgor pressure renders the chemical potentials of intracellular and extracellular water equal at equilibrium. The cell plasmolyses when ΔP becomes negative.

II. Rationale for the accumulation of compatible solutes

After an osmotic upshift, cells benefit from the accumulation of compatible solutes by balancing the osmotic disturbance via an increase in cytoplasmic osmolality. High intracellular concentrations of compatible solutes are common under high osmolality conditions, but it is difficult to derive a quantitative relationship between the internal concentrations of osmolytes and the external osmolality as multiple (macro)molecules are involved. The solutes to be accumulated to high

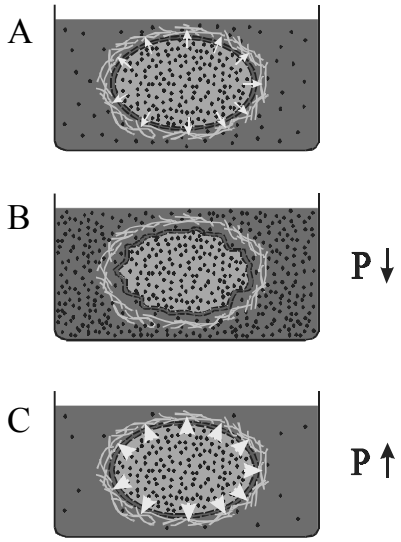

Fig. 1. Bacteria can be subjected to osmostatic conditions (A), an osmotic upshift (B) or an osmotic downshift (C). The arrows indicate the size/direction of the turgor pressure (P).

intracellular levels are restricted to a few categories (for reviews see Csonka, 1989 (21); Csonka and Hanson, 1991 (22); Galinski, 1995 (40)), and they include (i) potassium ions, (ii) amino acids (glutamate, alanine, proline), (iii) amino acid analogues (taurine, N-acatylglutaminylglutamine amide), (iv) methylamines and related compounds (glycine betaine, carnitine, ectoines), (v) polyols and sugars (glycerol, sucrose, lactose). Although K^+ offers osmoprotection, it is by definition not a compatible solute. In general, compatible solutes should not interact specifically with the (mostly negatively charged) cellular macromolecules, nor should they perturb cytoplasmic solutes via (de)hydratation, precipitation, or any other (charge) interaction. Under *steady state* osmotic conditions, compatible solutes, present in large amounts in the cytoplasm, have no net charge.

Both Gram-positive and Gram-negative bacteria prefer particular zwitterionic organic cosolvents such as glycine betaine, carnitine and ectoine as osmoprotectants (157). In enteric bacteria, the accumulation of potassium ions in response to an osmotic upshift is usually only transient and is followed by the synthesis of other compatible solutes (e.g. trehalose), and the induction of uptake systems for glycine betaine

and proline, eventually leading to the replacement of potassium by these solutes (see also Poolman and Glaasker, 1998). Accumulation of glycine betaine not only restores turgor following an osmotic upshift, but also increases protein stability. Glycine betaine has proved to stabilize enzyme structure and, in special cases, may serve a role as low molecular weight chaperone (5;12;31). The stabilization of native protein structures by compounds like glycine betaine involves preferential exclusion of the solute from the protein's surface, which increases the protein chemical potential, leading to preferable hydration of the macromolecules. The preferential exclusion implies that the interaction between protein and compatible solutes is thermodynamically unfavorable.

III. Structural basis for osmosensing

1. Mechanosensitive channels are involved in the response to an osmotic downshift

Mechanosensitive channels are involved in the response to osmotic downshifts by expelling solutes when the turgor becomes too high. The best-studied system is the MscL protein from *Escherichia coli*, of which homologues are present in most, if not all, bacteria. A crystal structure at 0.35 nm resolution of the MscL protein, in the closed state, from *Mycobacterium tuberculosis* is available (19;142;143). This structure has formed the basis for modeling and experimental studies and yielded information about the open state. MscL is a homopentamer consisting of two transmembrane α-helices per subunit, with both termini located at the cytoplasmic side of the cytoplasmic membrane. The five amino-terminal amphipathic α-helices have been postulated to form a cytoplasmic gate, which is pulled apart when the transmembrane domains expand with increasing membrane tension (141).

Upon gating, MscL jettisons out solutes with little discrimination, except for size. Apparently, even small proteins, which are detected in the medium subsequent to osmotic downshock, can leave the cell through this channel (1). MscL, with its huge pore (conductance of 3.6 nS and estimated pore of 30 to 40 Å in diameter) appears to be a final resort to release high pressures resulting from acute external osmotic downshifts. The reduction of osmotic pressure of the cytoplasm at less severe hypo-osmotic stresses seems to be mediated by other mechanosensitive channels (*e.g.* MscS in *E. coli*, which is more sensitive to membrane tension and only 1 nS in conductance) and the activation of solute-specific efflux systems (84;110).

The increase in tension in the membrane, following water influx, may be sensed as a decrease in overall pressure on the protein, which would facilitate the transition from the closed to the open state. The accompanying membrane thinning and the resulting hydrophobic mismatch may also contribute to the stability of one or more of the proposed conformational states (50).

Homologues from several species have been shown to encode channel activity when expressed in *trans* in *E. coli*, strongly suggesting that they are orthologues (99). Interestingly, at least one marine bacterial species, *Vibrio alginolyticus*, does not appear to contain a MscL and is sensitive to osmotic downshift, while expression of the *E. coli* MscL in *trans* allows this cell to survive such a challenge (101).

2. Transport systems are involved in the response to an osmotic upshift

2.1. Primary transport systems

The primary transport systems involved in the response of bacteria to an osmotic upshift are substrate-binding protein-dependent ABC transporters, which use ATP as source of energy to drive solute translocation against the concentration gradient. Such transporters are typically composed of five protein(s) (domains), that is, an extracellular substrate-binding protein, two ATP-binding/hydrolyzing subunits and two integral membrane subunits (Fig. 2). Except for the substrate-binding protein, the

other subunits can be present as distinct polypeptides or fused to one another but always each entity is present twice. The substrate-binding protein scavenges its surroundings for a ligand and, subsequently, binds and delivers it to the integral membrane subunits that accommodate the translocation pathway. Upon association of the binding protein with the membrane complex, ATP-hydrolysis by the two ATPase subunits provides the energy needed for both substrate translocation and resetting of the system for another transport cycle. The osmoregulated ABC transporters belong to the OTCN family (26), of which the members transport substrates as diverse as quaternary ammonium compounds (glycine betaine, carnitine), proline, alkyl-sulfonates and -phosphonates, phosphites, cyanate, and nitrate.

Although biochemical evidence is not available, the osmoregulated ABC transporters most likely have two identical copies of the ATP-binding subunit. This suggestion is based on the finding that the operons specifying osmoregulated transporters only have a single gene for an ATP-binding subunit. The integral membrane components are either present as two identical copies or two homologous proteins, but they are not fused to each other nor to the ATP-binding subunit. The ligand-binding subunit is present as a free protein in the periplasm of Gram-negative bacteria, whereas in Gram-positive bacteria the protein can be anchored to the membrane through a fatty acid modification of the amino-terminal cysteine (73;131). Recently, it has been shown that a subset of ABC transporters has the ligand-binding protein fused to the integral membrane subunit (this thesis). OpuA of *Lactococcus* lactis is the only well-studied representative of this subset of binding-protein dependent ABC transporters, but database searches indicate that similar systems are present in a range of Gram-positive organisms but also in the Gram-negative bacterium *Helicobacter pylori* (this thesis).

By analogy with other ABC transporters, functional OpuA will most likely be composed of two integral membrane subunits and two

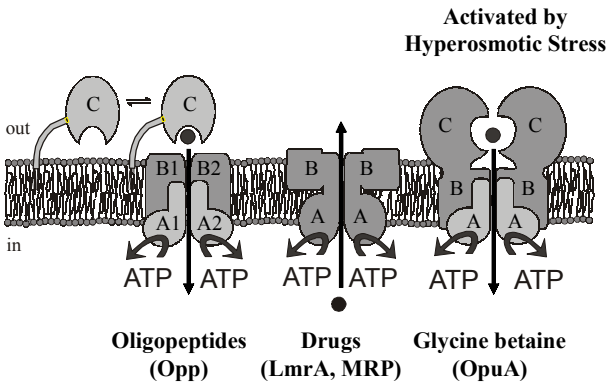

Fig. 2. Architecture of ABC transporters. The membrane-embedded transporters are composed of ATPase subunits (A), translocator subunits (B) and substrate-binding proteins/domains (C).

ATP-binding subunits. Since the translocator subunit is fused to the substrate-binding protein (OpuABC subunit), the oligomeric structure implies that two receptor domains are present per functional complex (Fig. 2). This raises questions about the observations that only a single substrate-binding protein interacts with the dimeric membrane complex of an ABC transporter, and that two lobes of a single substrate binding protein interact with different integral membrane protein(s) (domains) (36;87).

2.1.1. The ABC component

The ABC- or ATPase component of known osmoregulated ABC transporters has a molecular mass of about 45 kD. Similar to the ATPase subunit (MalK) of the maltose transporters of *E. coli* and *Thermococcus litoralis* (32), the ABC component of most osmoregulated systems consists of an amino-terminal α/β type ATPase domain (~27 kD) and a carboxyl-terminal regulatory domain (~18 kD). The carboxyl-terminal domain in MalK of *E. coli* provides the system with a means to control the transport activity as well as the expression of the *mal* genes through interaction of MalK with regulatory proteins. Whether the regulatory domain of the ATPase of the osmoregulated ABC transporters has such a role is not known. Database searches indicate that some homologues of the family of osmoregulated ABC transporters have an ATPase component with a truncated regulatory

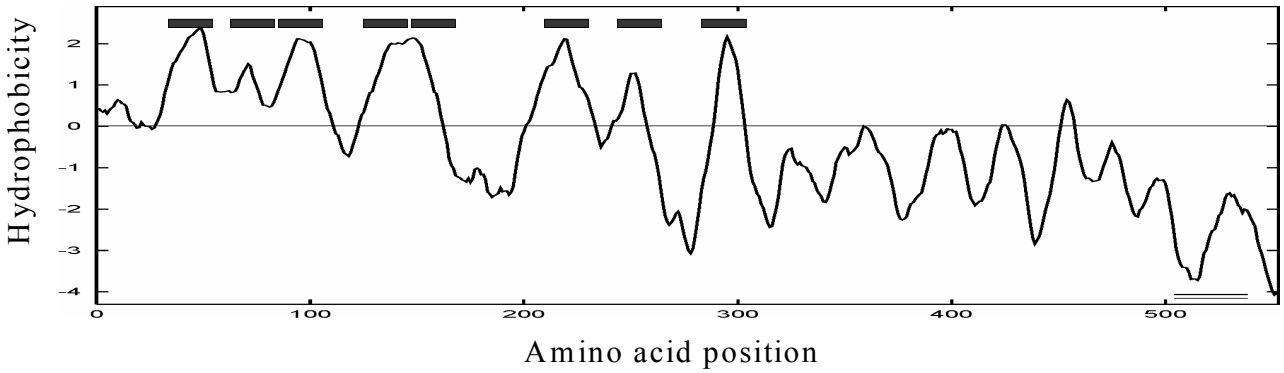

Fig. 3. Hydropathy plot of OpuA. The bars indicate α-helical transmembrane segments.

domain. These systems, *e.g.* ChoQ of *L. lactis*, have a molecular mass of about 35 kD.

2.1.2. The integral membrane component

Hydropathy profiling of the sequences of the integral membrane components of the osmoregulated ABC transporters shows that the systems fall in four subfamilies (Fig. 3 and 4). Each of the transporters has the conserved EAA motif in the equivalent cytoplasmic loop (loop V-VI in subfamilies I and III and loop III-IV in subfamilies II and IV). These EAA regions are thought to constitute a recognition site for the helical domain of the ABC component (134). Members of subfamily I, represented by ProW of the *E. coli* ProU system and OpuAB of *Bacillus subtilis* OpuA, are predicted to have seven transmembrane α-helical segments with the N-terminus facing the external surface of the membrane and the C-terminus facing the cytoplasm. This membrane topology is supported by *phoA* and *lacZ* fusions, albeit a limited set of data (49). ProW differs from OpuAB (and other subfamily I homologues) by having an unusually long amino-terminus of about 100 residues, which protrudes into the periplasmic space of *E. coli*.

The members of subfamily II, represented here by OpuCB and OpuCD of the OpuC system of *B. subtilis*, have five transmembrane α-helical segments with the N- and C-termini at the external and internal side of the membrane, respectively. Thus, compared to the members of subfamily I, the first two transmembrane segments are missing.

Subfamily III, exemplified by OpuABC of the OpuA system of *L. lactis,* is similar to subfamily I except that the proteins have the substrate-binding domain fused to the integral membrane component. In this case, an eight transmembrane segment allows the binding domain to face the exterior of the cell. It is worth noting that the majority of multidomain membrane transport proteins have the individual domains fused to another via so-called flexible linker regions (146). In the case of OpuA of *L. lactis*, the predicted end of the eight transmembrane segment is followed by a sequence that over its entire length is highly similar (>50% identity) to the ligand binding protein ProX of the *E. coli* ProU system. On the assumption that the amino-terminus of ProX forms an intrinsic part of the binding protein, a flexible linker will not be present between the transmembrane and ligand binding domains of the OpuABC polypeptide of the OpuA system (this thesis).

Finally, members of subfamily IV, exemplified by ProWX of *H. pylori* and the membrane component of the ChoQ complex of *L. lactis*, are similar to those of subfamily II, except that also in this case the ligand-binding protein is fused to the integral membrane component. This gives rise to a membrane protein with six predicted transmembrane α-helical segments and an amino- and carboxyl-terminus that are facing the outer surface of the membrane. At present, it is unclear whether the smaller sizes of the proteins of subfamilies III and IV are related to a different functioning of

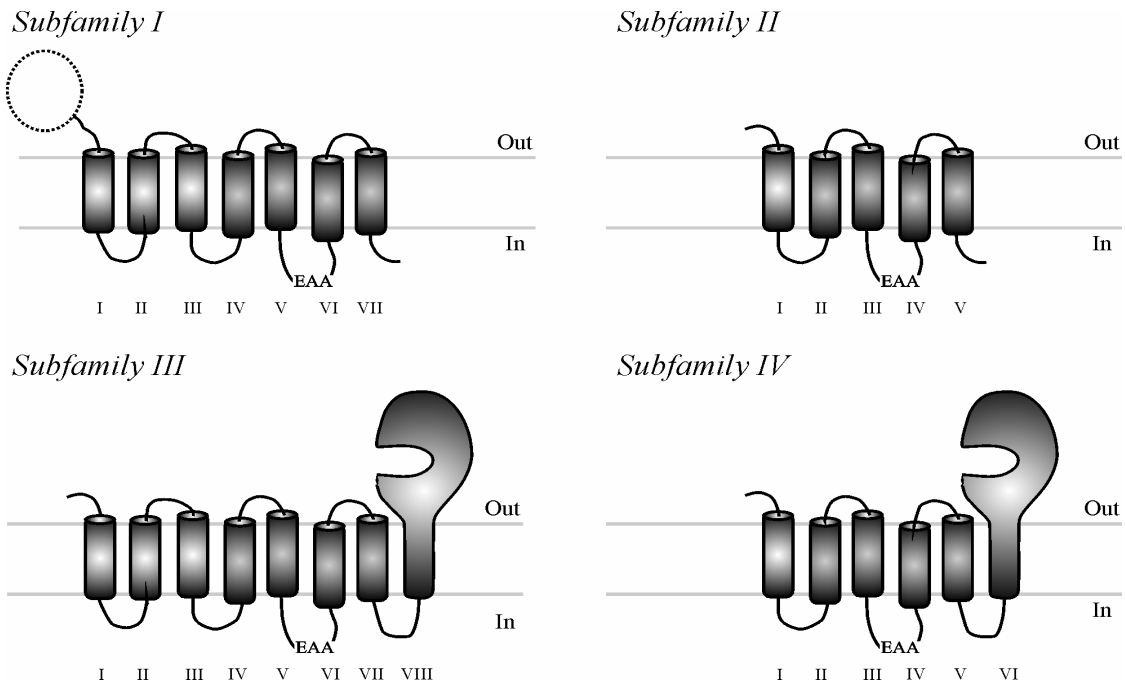

Fig. 4. Topology models of the members of the four subfamilies of osmoregulated ABC transporters. The additional periplasmic domain in some members of subfamily I is indicated as dotted circle. The packman-like structure connected to the last transmembrane segment of the members of subfamilies III and IV represents the ligand-binding domain. The EAA motif in the cytoplasmic loop is also depicted.

the systems when compared to those of the subfamilies I and II.

2.1.3. The substrate-binding protein

Members of subfamily I and II have a soluble substrate-binding protein that in Gram-negative bacteria resides in the periplasm. In Gram-positive bacteria, the "soluble" binding protein of subfamily I and II type transporters is anchored to the outer surface of the cytoplasmic membrane via an amino-terminal lipid moiety. Recently, ProX of *E. coli* has been crystallized (13) and the structure, although not published, has been presented at scientific meetings. As expected ProX has an overall tertiary fold typical for ligand binding proteins belonging to ABC transporters (116), which is indicative for a Venus fly-trap mechanism for substrate binding. Interestingly, the substrate, glycine betaine, is bound to Trp residues via cation-π interactions similar to the observed binding of quaternary ammonium compounds to acetyl-choline esterase of human brain (6)(Bremer and Welte, unpublished). The π-electrons interact with the quaternary ammonium group of glycine betaine. One of the three Trp residues that interacts with the substrate is highly conserved in the glycine betaine binding proteins for which the primary sequences are available to date.

It has been proposed that the interaction of the liganded substrate-binding protein with the membrane complex transmits a signal, via the membrane-spanning membrane proteins, to the ABC subunits on the other side of the membrane, thereby triggering ATPase activity and translocation of the substrate molecule. For the maltose ABC transporter from *E. coli* it has been reported that the maltose binding protein (MBP) binds substrate with a K_d that reflects the K_m of transport, but this is not a property of all substrate-binding protein-dependent ABC transporters (83). The substrate-binding protein is the main determinant of substrate specificity of substrate-binding protein-dependent ABC transporters (95).

2.2. Secondary transport proteins

This type of transporter uses the electrochemical solute and/or ion gradient across the membrane to drive translocation of substrate. Transport is bi-directional, depending on the direction of the electrochemical gradients of the translocated species. Well-studied osmoregulated secondary transport proteins are ProP from *E. coli* and BetP from *Corynebacterium glutamicum*. On the basis of predictions of the secondary structure, it is assumed that these systems are composed of 12 α-helical transmembrane segments with both the N- and C-terminus located at the cytoplasmic side of the membrane (24;108). The BetP protein has amino- and carboxyl terminal extensions that are not commonly found in secondary transport proteins. Truncations in the negatively charged N-terminus of BetP resulted in a shift of the maximal activation to higher osmolalities, while deletions in the positively charged C-terminus resulted in the complete loss of the osmoregulated transport activity (108).

The ProP protein is differentiated from the majority of homologues in the MFS family by its C-terminal extension, which is predicted to form an α-helical coiled-coil (25). For a synthetic polypeptide corresponding to the C-terminus of ProP, it has been shown that it forms a dimeric α-helical coiled-coil. Point mutations in the peptide, equivalent to those that altered the activation profile of the wildtype ProP protein, affected the oligomeric state of this α-helical coiled-coil structure. A C-terminal truncated derivative of ProP is inactive, implying a direct role of the C-terminal domain in the osmotic activation of the transporter. The C-terminus could be involved in the formation of a homo- or a hetero-oligomeric coiled-coil, resulting in self-association or association with cytoplasmic proteins or membrane-associated proteins. A possible role for a cytoplasmic component is suggested by the finding that the soluble protein, ProQ, is required for full activation of ProP *in vivo* (79). This structural element did not affect the transcription or translation of ProP, implying that ProQ influences the osmotic activation of ProP at a posttranslational level. Although direct evidence is lacking, it has been speculated that ProQ interacts with the C-terminal domain of ProP, thereby influencing the stability of the coiled-coil structure and the active conformation of the transporter (25).

IV. Specificity and transport mechanism of ABC transporters

1. Substrate specificity of osmoregulated transport proteins

The substrate specificity of osmoregulated (ABC) transporters has been investigated most extensively in *B. subtilis* (72). Some systems seem very specific, *e.g.* OpuB only selects choline, whereas others such as OpuA accept a wide range of substrates. It should be stressed, however, that in neither case the specificity has been determined directly, that is, as dissociation constants (K_d-values) of ligand binding to the receptor. The specificity of bacterial osmoregulated ABC transporters has been determined either as apparent affinity constants of transport (K_m) or it has been inferred from the ability of a compound to offer protection during growth under hyperosmotic conditions. The differences in narrow versus broad specificity are not readily revealed in the primary sequences of the ligand binding proteins/domains when analyzed by multiple sequence alignments. Generally, osmoregulated transporters involved in the uptake of methyl-amines and related compounds (e.g. glycine betaine, carnitine) have an high affinity for these substrates (low micro-molar range).

2. Directionality of transport by ABC trans-porters

From a thermodynamic point of view, the hydrolysis of one ATP molecule per molecule of translocated substrate would be more than sufficient to explain the observed accumulation ratios. For instance, a $\Delta G_{ATP}/F$ of -500 mV,

typical for growing cells, would allow a more than 10^8-fold concentration of substrate with a reaction stoichiometry as low as 1. The observed accumulation ratios are much lower and the systems operate far from dynamic equilibrium. This has led to the widespread acceptance that ABC transporters function unidirectionally. However, for three amino acid ABC transporters, the general amino acid permease (AapJQMP) and branched-chain amino acid permease (BraDEFGC) from *Rhizobium legiminosarum*, and the histidine permease (HisJQMP) from *S. typhimurium,* it has been reported that substrate transport is bi-directional (58). The Bra system belongs to the hydrophobic amino acid uptake transporter (HAAT) family, while the His and Aap systems belong to the polar amino acid uptake transporter (PAAT) family. In whole cell experiments, evidence has been obtained for both substrate-binding protein-dependent uptake and efflux. The uptake and efflux of substrate seem to be independent processes, that is, efflux is not stoichiometric with incoming solute. The net substrate accumulation-level depends on the rate of uptake and efflux, and a plateau is reached when both processes are saturated with substrate. The K_m for uptake is 10^3-10^4 lower than that for efflux but the V_{max} of both processes is similar (58).

The efflux of substrate is not observed when the HisJQMP complex is characterized in proteoliposomes. However, maximal attainable histidine concentration inside the proteo-liposomes was only 230 μM (85), which may have been too low to saturate the efflux pathway. Notice that the Aap system has a K_m for efflux of substrate of 10.8 mM (58). Given the conservation of structure of ABC transport systems, bidirectional solute movement may be a more general feature of this superfamily than recognized thus far (58). Additional studies have to be performed to show that the bi-directional transport is not an artifact of the *in vivo* experimental system. When subjected to an osmotic upshift, the osmo-adaptive response of microorganisms involves the accumulation of compatible solutes (e.g. glycine betaine) to (sub-)molar concentrations. It is hard to envision that ABC transporters involved in the accumulation of such solutes display bi-directional transport with a K_m for efflux of solutes in the millimolar range. It cannot be excluded, however, that osmoregulated ABC transporters facilitate efflux of solutes at higher intracellular concentrations with K_m-values for efflux in the (sub)-molar range.

3. Energy-coupling of ATP hydrolysis driven transport

One of the major unsolved questions in the field of ABC transport proteins relates to the reaction-stoichiometry, that is, how many molecules of ATP are hydrolyzed per molecule of substrate transported. Well-studied substrate-binding protein-dependent ABC transport systems are the maltose transporter from *E. coli* and the histidine transporter from *S. typhimurium*. For the partially purified and membrane-reconstituted histidine and maltose transporters, a stoichiometry of 5 and between 1.4-17 has been reported, respectively (7;28). These apparent stoichiometries cannot be understood in mechanistic terms. As many as two ATP molecules seem more plausible because ABC transporters have two ATP-binding domains.

For the purified and membrane-reconstituted histidine transporter, it has been suggested that the spatial relationship between the two lobes of the substrate-binding protein is one of the determinants in the association with the transmembrane complex that dictates the efficiency of translocation. This spatial relationship between the lobes would depend on the type of substrate bound. Indeed, the apparent relative ratio of ATP molecules hydrolyzed per substrate molecule translocated is 1.0 for L-histidine, 0.46 for L-arginine, and 0.17 for L-lysine (87). However, it cannot be excluded that the contribution of efflux to the overall transport differed for the various substrates (as discussed in the previous section), which would lead to apparent differences in the energy coupling efficiencies. Furthermore, the

unliganded substrate-binding protein assumes with low frequency a closed state, referred to as "breathing" of the protein, allowing it to interact with the membrane complex in a manner that stimulates a low rate of ATP hydrolysis (4). This intrinsic ATPase activity also influences the observed ATP/substrate stoichiometry.

Despite the fact that substrate-binding protein-dependent ABC transporters use accessory proteins to bind substrate, it is likely that the essential features of the transport mechanism are conserved throughout the whole ABC-superfamily. In this respect, it is of interest to consider also the solute efflux proteins, e.g. the multiple-drug resistance (MDR) transporters. Well-studied ABC-type efflux systems are the human multidrug transporters P-glycoprotein (150a) and MRP1 (84a), and the bacterial multidrug transporter LmrA (151a). These transporters remove hydrophobic drugs from the cell by flip/flopping them from the inner to the outer cytoplasmic membrane leaflet, or by expelling the drugs from the inner leaflet directly into the outside medium. On the basis of biochemical data, it has been proposed that two molecules of ATP are hydrolyzed in a single turnover of the catalytic cycle of P-glycoprotein, one in the transport of substrate and the other in effecting conformational changes to reset the pump for the next catalytic cycle (127;133). For P-glycoprotein and LmrA, it has been shown that the hydrolysis of the first ATP molecule is accompanied by the loss of high-affinity drug binding, which presumably facilitates translocation of the drug from the inner to the outer membrane leaflet (132;152). This switch from high- to low-affinity may be analogous to the one occurring in substrate-binding protein-dependent ABC transporters. For the maltose transporter from *E. coli*, it has been shown that the complex can be locked in a state following hydrolysis of the first ATP molecule, which is achieved by the action of the phosphate-analog vanadate (Fig. 5). Vanadate exchanges with the free phosphate-group that is formed in the hydrolysis of ATP, thereby trapping ADP in the nucleotide-binding site. Interestingly, the maltose-binding protein (MBP) is tightly associated with the membrane-embedded proteins in the vanadate-inhibited complex. As maltose was absent from this complex, it has been suggested that maltose is released from MBP prior to formation of the MBP-translocator complex. MBP has an open and a closed conformation with a 'low'- and a 'high'-affinity for maltose, respectively. Thus, it is conceivable that MBP is in a conformation that resembles the open conformation when it is tightly bound to the membrane transporter, thereby reducing its affinity for maltose (20). On the basis of the above-mentioned data, it has been proposed by Chen *et al.* that the hydrolysis of ATP is coupled to conformational changes in the transporter complex. These eliminate the high-affinity sugar binding to MBP in the transporter complex, which facilitates the transfer of the sugar molecule across the membrane.

A second feature that is conserved among the members of the ABC superfamily is the cooperativity between the two nucleotide-binding sites (20;86;136). In both P-glycoprotein and the maltose transporter, trapping of ADP by vanadate in one nucleotide-binding site inhibits the ATPase activity of the other site (20;132). Several crystal structures of nucleotide-binding subunits are available, *e.g.* HisP from *S. typhimurium* (59), MalK from *T. litoralis* (32), MJ1267 from *Methanococcus jannaschii* (70), and the mammalian TAP1 protein (41). The HisP and MalK proteins share a similar architecture as expected from the sequence homology, and the nucleotide-binding site is highly exposed on the surface of the monomer unit, raising the possibility that residues from another subunit in the transport complex may contribute to binding and/or hydrolysis of ATP (62). The proposed dimer, while different from the dimer seen in the crystal structure of HisP (59), is strikingly similar to a recently published dimer of the RAD50 catalytic domain (RAD50cd), an ABC-protein involved in double strand break repair (57). ATP promotes the association of two inactive RAD50C proteins by binding to the

signature motif (or LSGGQ) of one monomer and the P-loop (or Walker A motif) of the opposite monomer to complete the active site, thereby activating the ATPase activity (57). This display of cooperativity is consistent with the recent observation that the activation of ATPase activity in the maltose transporter is associated with conformational changes that bring the two MalK (ATPase) subunits closer together (91). These data are readily interpretable by assuming that ATP is bound between the two MalK subunits in $MalFGK_2$ and that the association and dissociation of the MalK subunits regulates the ATPase activity of the transport complex (Fig. 5) (91). The mechanism of coupling transport to hydrolysis of ATP in bacterial ATP-binding cassette transporters has recently been reviewed by Davidson (27).

The mechanism of substrate-binding protein-dependent ABC transporters has been summarized in a six-step model of the catalytic cycle, which to a large extent originates from studies on the histidine transporter ($HisJQMP_2$) from *S. typhimurium* (88). In the resting state (absence of substrate), both HisP molecules (ATPases) are tightly associated to the integral membrane complex, which consists of HisQM. In this state, HisP is prevented from hydrolyzing ATP. In the first step, ATP binds to one of the HisP molecules, resulting in a conformational change and partial disengagement of the HisP dimer from the membrane complex. In the second step, the initial binding of ATP to the first HisP molecule cooperatively promotes the binding of a second ATP molecule to the other HisP subunit. The partial disengagement of the HisP molecules from the membrane complex releases some of the ATPase suppression, enabling intrinsic ATPase activity, and priming the permease for the translocation of a substrate molecule, which is dependent on ATP-hydrolysis. In the third step, the liganded substrate-binding protein, HisJ, interacts with the periplasmic face of the membrane complex, resulting in the further disengagement (association) of the HisP molecules and triggering of the hydrolysis of the first ATP

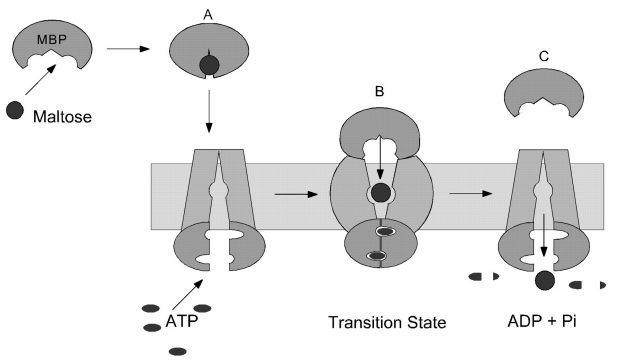

Fig. 5. Model for maltose transport. (A) MBP binds maltose, undergoing a change from an open conformation to a closed conformation, generating a high-affinity sugar-binding site. In the closed conformation, MBP binds $MalFGK_2$ to initiate transport and hydrolysis. (B) In the transition state for ATP hydrolysis, MBP becomes tightly bound to $MalFGK_2$, and internal sugar-binding sites are exposed to each other. This opening of MBP in the transitions state reduces the affinity of MBP for maltose, facilitating the transfer of sugar to $MalFGK_2$. (C) Maltose is transported, and MBP is released after reexposure of the membrane-binding site to the cytoplasm. MBP activates the ATPase activity of MalK by bringing the two MalK subunits into close proximity, completing the nucleotide-binding site(s) at the MalK interface with residues donated from the opposing subunit. Reprinted from reference (20).

molecule. In step four, subsequent conformational changes in $HisQMP_2$ open the translocation pathway, allowing the passage of a solute molecule. The first P_i may also be released at this stage. In step five, the first ADP molecule is released and ATP hydrolysis occurs at the second site. A second ligand bound to the extracellular solute receptor is now released. This second solute molecule may have bound to the substrate-binding protein without the receptor being released from the membrane complex (103). However, the translocation of a second substrate molecule remains disputable, as there is no solid information on the stoichiometry between hydrolysis and translocation. In step six, the second ADP and P_i are released and the unliganded substrate-binding protein leaves the complex, resetting the transporter to the initial resting state. The release of the substrate-binding protein does not necessarily have to be complete as liganded and unliganded binding-proteins interact with equal affinity to the membrane complex (4). In

contrast to the liganded binding-protein, the unliganded binding-protein only induced a low ATP-hydrolyzing activity of the ATPase-subunit (HisP) (4), which is associated with the membrane complex. These findings challenged the conventional model in which there is a switch in affinity from high to low of the receptor for the membrane complex upon transfer of the substrate. It has been proposed that liganded and unliganded binding-proteins interact differently with the membrane complex, even though the apparent affinity constants for binding are similar. In this model, the de-liganded binding-protein would have a tendency to remain associated with the membrane complex to capture free substrate for sequential cycles of translocation, a situation predicted to occur permanently in OpuA and homologues that have the substrate-binding protein fused to the translocator.

V. Osmoregulation at the level of expression and transport activity

Most osmotically controlled uptake systems are regulated both at the genetic (induction of the genes) and the enzymatic level (direct "activation" of the transport protein). The primary response to osmotic stress involves the (in)activation of transporters which are already present in the membrane, as synthesis of these systems would take too long to effectively cope with osmotic challenges. The degree of induction can vary considerably (from 2 up to 500-fold), whereas *in vivo* activation of the transport protein by an osmotic upshift is usually in the range of 5 to 35.

A major topic in the field of cell volume regulation concerns the identification of the osmotic signal that triggers the response to osmotic stress and the unraveling of the mechanism(s) rooting the osmotic activation of osmoregulated transporter, channel and signal transduction proteins. The following section deals with the osmotic regulation of gene expression and transport activity, and potential osmotic signals will be discussed. For general aspects of osmoregulation and mechano-sensation in bacteria, we refer to previous reviews by Booth and Louis (10), overview of how a cell manages osmotic downshift conditions; Csonka and Hanson (22), possible mechanisms underlying osmoregulatory responses; Hamill and Martinac (50), molecular basis of mechanosensation; Poolman and Glaasker (110), overview of how a cell regulates pool sizes for compatible solutes; and Wood (157), most comprehensive and general review on osmoregulation in bacteria.

1.1 Osmotic regulation of gene expression of osmoregulated transporters

The osmoregulated transporter ProU from *E. coli* is the best-studied ABC transporter in terms of osmotic regulation of gene expression, but despite intensive efforts, a full understanding of the molecular mechanism(s) underlying *proU* expression has not yet been achieved. The kinetics of induction of the *proU* operon involves a lag phase of 15 to 20 min., followed by a rapid increase in expression, and, subsequently, a slow decay in the expression rate (64). Therefore, the increased expression of *proU* could be mediated by signalling molecules (second messengers) that have to build up in the cytoplasm, rather than by direct activation of signal transduction pathways. Consistent with a role for specific second messenger molecules are observations that potassium-glutamate is (at least partially) responsible for the induction of *proU* (9;121), but other studies have rejected these claims (23;65). It is now thought that the stimulation of transcription of *proU in vitro* by K^+-glutamate is a manifestation of its favorable effect on macromolecular function (*e.g.* RNA polymerase-promoter interaction) and not unique to osmotic regulation of the *proU* promoter (23). Taken together, the data are consistent with intracellular osmolality as signal for *proU* transcription, which would lead to maximal levels of ProU at a time that the turgor has already (largely) been restored. Such a signaling mechanism is line with the observation that *E. coli* responds to an osmotic upshift by rapidly accumulating K^+-glutamate

and subsequent replacement of these ionic osmolytes for neutral ones such as the substrate (glycine betaine) of the ProU system.

1.2. Transcription factors and osmoregulated promoters

Contrary to what one would expect for a system that is tightly regulated by the medium osmolality (>500 fold induction), specific transcription factors do not seem to be involved or at least they have not been discovered. Genetic searches for such proteins have led to the isolation of mutants with defects in general DNA-binding proteins such as TopA, GyrAB, IHF, HU and H-NS (72). Mutations in these proteins have pleitropic effects on gene expression, for instance, through alterations in DNA supercoiling, and it is unlikely that the tight osmotic control of the *proU* operon is solely mediated by these proteins. On the basis of transcriptional analysis of the P1 promoter of the *proP* gene, which encodes an osmoregulated secondary proline transporter, the suggestion has been made that the cAMP receptor protein (CRP) could function as a general osmoregulator of transcription in *E. coli* (82). Binding of CRP to a site within the *proP* P1 and some other promoters is destabilized after an osmotic upshift. These studies imply that CRP could have a general osmoregulatory role in addition to its function in catabolite control.

Transcription of *proU* is effected via the promoters P1 (sigma factor σ^S) and P2 (sigma factor σ^{70}). During exponential growth, transcription from P2 contributes most to the expression by employing σ^{70}. Sigma factor σ^S generally contributes to the expression of genes in the stationary phase of growth, but the transcription of *proU* is not significantly increased under those conditions. The presence of potassium-glutamate enhances the transcription via σ^S and σ^{70}, and increases the selectivity of σ^S for P1 *in vitro* (120). Additionally, recent work from Rajkumari *et al.* suggests that the P1 promoter is involved in the expression of the *proU* operon during cold stress (119). In line with this observation, the authors note that the *proU* operon in *E. coli* and *Salmonella enterica* mediates adaptive accumulation of glycine betaine in response to both osmotic and cold stress and that the P2 and P1 promoters are primarily responsible for transcription of the operon under the respective conditions. This would be consistent with an overlap between the adaptation to osmotic and cold stress as has been proposed earlier in plants and other bacteria (137;162).

In organisms other than *E. coli*, *e.g. B. subtilis* and *L. lactis*, the expression of the genes specifying osmoregulated ABC transporters is also under osmotic control (52;72), but little is known about the signals and transcription factors that regulate the expression. In *B. subtilis,* a general stress regulon is present whose expression depends on the alternative sigma factor SigB (σ^B). Besides salt, also heat, oxidative and pH stress affect the expression of the σ^B regulon (158). The induction of the σ^B regulon by osmotic upshift is only transient and σ^B-controlled proteins cannot adequately protect cells against prolonged high osmolality stress. The structural genes for the glycine betaine (OpuD) and proline (OpuE) secondary transport proteins are members of the σ^B regulon, but there is no evidence that the osmoregulated ABC transporters (OpuA, OpuB and OpuC) of *B. subtilis* are under the control of σ^B. The choline-specific transporter OpuB is under the control of the GbsR repressor, but this transcription factor is a choline sensor rather than an osmosensor/regulator (14). Finally, it should be stressed that maximal rates of uptake via OpuA and OpuC of *B. subtilis* increase only 1.5 to 3-fold when 0.4 M NaCl is added to the growth medium (69), indicating that the corresponding genes are not under tight osmotic control as for instance *proU* of *E. coli*. A preliminary report on the regulation of expression of the ABC transporter OpuA of *L. lactis* suggests that a transcriptional regulator of the GnrR family acts as a repressor of the *opuA* operon (47), but the signal sensed by the protein is not known.

2. Osmotic regulation of activity of osmoregulated transporters

2.1. Potential osmotic stress-related signals

Osmotic activation of membrane proteins may be signaled via: (i) a change in cell turgor; (ii) mechanical deformation of the membrane (macroscopic change in membrane structure); (iii) mechanical stimulus originating within the exo- or cytoskeleton of the cell; (iv) a change in the hydration state of the protein (internal or external osmolality); (v) alterations in the physicochemistry of the membrane bilayer (protein-lipid interactions); (vi) a change in cytoplasmic ion concentration or ionic strength; (vii) specific molecules interacting directly with the protein (Fig. 6). With regard to the physicochemistry of the membrane, a number of properties are affected as a function of osmotic shift, including membrane fluidity, bilayer thickness, hydration state of lipid headgroups, and interfacial polarity and charge. Each of these parameters contributes to the lateral pressure of a membrane. The lateral pressure is composed of the components of the interactions between the various membrane constituents, specifically the derivative of their free energy with respect to area. The lateral pressure profile is the depth-dependent distribution of lateral stresses within the membrane (16), a global parameter that is often discussed in terms of "intrinsic strain", "intrinsic curvature", "curvature strain" or "internal tension" (46;92;157). In this paper, we use lateral pressure profile as description of the lateral stresses in lipid membranes. The relevance of each of the physicochemical parameters of the membrane to osmosensing and the available evidence for a particular mechanism are described in the following sections.

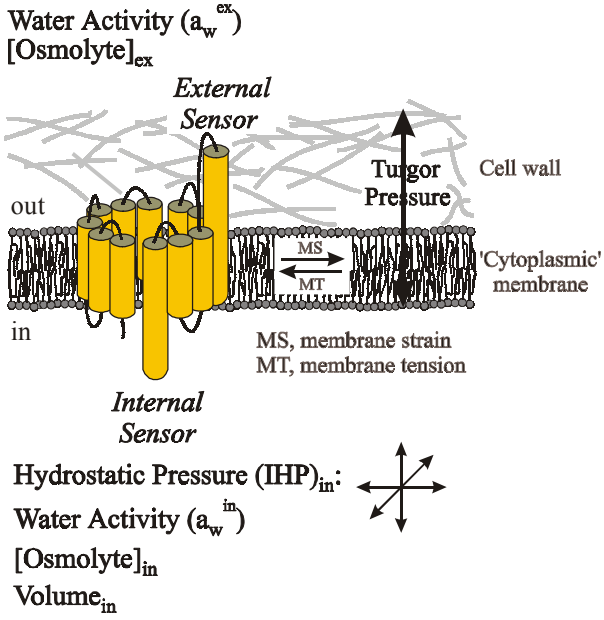

Fig. 6. Potential osmotic stress-related signals.

2.1.1. Turgor

Although cell turgor is required for the expansion of the cell wall, there is little information on what the lower limit of turgor should be before cell growth ceases. Depending upon the species, a bacterial cell may develop several to a few tens of atmospheres of pressure across the cell envelope. For a given organism, cell turgor will vary in response to increases or decreases in external osmolality. In *E. coli* the turgor decreases from ~3 to 0.5 atm when the osmolality of the growth medium is increased from 0.03 to 0.8 osm/kg (17). Although a turgor of 0.5 atm may be sufficient to sustain growth of *E. coli*, it is well possible that the lower limit of turgor of Gram-positive bacteria, with their generally thicker cell wall, is much higher. In theory, osmosensors could detect changes in cell turgor. In fact, such a model has been proposed for the membrane-bound sensor kinase KdpD, a sensor that osmotically regulates expression of the *E. coli kdpFABC* operon, which encodes a P-type potassium-ATPase (80). This model was rooted in the assumption that turgor is maintained across the cytoplasmic membrane. However, there is evidence that in Gram-negative bacteria, the cytoplasm and periplasm are isotonic and that turgor is maintained across the cell wall-outer membrane complex rather than cytoplasmic membrane (17). If true, turgor would not likely be the primary regulatory signal for transporters or signal transduction components present in the cytoplasmic membrane. Moreover, since membrane vesicles and liposomal systems can

only withstand low pressures as compared to cells with a peptidoglycan layer (22), functional incorporation into such artificial membranes of sensors that respond to low turgors should lead to constitutive activity. Several classes of osmoregulated systems, however, including the ABC transporter OpuA (this thesis), the ion-linked transporters BetP from *C. glutamicum* (129), ProP from *E. coli* (118), and the sensor kinases KdpD and EnvZ of *E. coli* (67;68) show normal functional regulation when incorporated into proteoliposomes or present in membrane vesicles, suggesting that turgor is not the salient stimulus. Together, these findings suggest that it is extremely unlikely that any bacterial osmosensing cytoplasmic membrane molecules respond directly to changes in cell turgor. The osmosensing devices of these systems must thus respond to the consequences of water influx or efflux, that is, changes in cytoplasmic solute concentration (osmolality), ionic strength, macromolecular crowding or physicochemical properties of the membrane in which the osmosensing proteins are embedded. This idea is consistent with recent *in vitro* studies on the sensor kinase KdpD, which, confusingly, is still described as being a turgor sensor (68).

The issue of whether mechanosensitive channels respond to pressure across the membrane, or to the tension within it, has been answered directly. One can determine if a channel is sensing membrane pressure or tension by imaging the curvature of a membrane patch, measuring the pressure across the membrane, and using Laplace's law,

$$t = p * r/2$$

in which *t*, *p* and *r* refer to tension, pressure and radius of curvature, respectively. Such analysis clearly demonstrated that MscL senses tension within the membrane, and not the pressure across it (144). Although the pressure required to gate the MscS channel has been observed to vary from patch to patch, it is always 2/3 of that required to gate MscL (Blount *et al*., 1999), strongly suggesting that this channel also responds exclusively to membrane tension. Finally, by measuring whole-cell currents in yeast cells, it has been demonstrated that mechanosensitive channels in this system are also gated by tension, suggesting that this mechanism may be conserved among pro- and eukaryotes (48). Hence, even for mechanosensitive channels it appears that these sensors do not sense cell turgor directly.

2.1.2. Hydration state

Substrate and ligand binding to enzymes and transporters is typically associated with changes in conformation of the proteins. Since it is likely that different protein conformations sequester different amounts of water, osmotic stress could potentially affect a systems activity through a change in hydration state of the protein. The classic example of water activity as a regulator of enzyme activity is hexokinase (106). The dissociation constant (K_d) for glucose binding to hexokinase decreases with increasing osmotic pressure of the assay medium, which has been varied with either low or high molecular weight polyethylene glycol (PEG) in the solution (124). The smaller effects of the low molecular weight PEGs are explained by their less effective steric exclusion from a cleft in the surface of the enzyme (see also hereafter). Similarly for the channel-forming peptide alamethicin, it has been shown that the open probability decreases with increasing concentrations of high molecular weight PEGs (155). There is no change in open probability when the water activity is varied with low molecular weight PEGs. Parsegian and colleagues (106) have formulated the thermodynamic basis for these observations. In going from the closed to the open state, the channel will need an extra amount of water as the open state is most likely more hydrated. Solutes too big to enter the channel such as high molecular weight PEGs will compete with the protein for water. Consequently, the excluded solute will cause the channel to perform extra osmotic work, which will lower the probability of the open conformation. The extra amount of work is less with solutes, *e.g*. low molecular weight PEGs,

able to enter the channel. In other words, low molecular weight PEGs have a less dehydrating effect on the protein than high molecular weight ones.

2.1.3. Ionic osmolytes and ionic strength

Upon an osmotic upshift the cellular volume is decreased, resulting in an increase in the concentration of intracellular ionic osmolytes. Such an increase in ionic strength, accompanying the volume decrease, is undesirable as too high concentrations of electrolytes interfere with macromolecular functioning in eubacteria as well as higher organisms (160). As best documented for *E. coli* (56;123), eubacteria expel ionic compounds in the event the electrolyte concentration becomes too high and replace these molecules with neutral osmolytes such as glycine betaine to balance the cellular osmolality. The increase in electrolyte concentration (or ionic strength) upon a modest decrease in turgor pressure could thus represent a trigger for the activation of any osmoregulated transporter for neutral compatible solutes. In this thesis (Chapter 5), we show that intracellular ionic osmolytes (or ionic strength) serve as signal to activate the ABC transporter OpuA.

Osmotic activation of the membrane-bound histidine kinase KdpD of the *kdpFABC* operon in *E. coli* similarly involves sensing of cytoplasmic ionic strength, but this system has additional, presumably, allosteric, mechanisms to sense K^+, as discussed in the next section (68;140). The sensor kinase EnvZ, reconstituted in proteoliposomes, is activated by K^+, Na^+, Rb^+ and NH_4^+, irrespective of the counterion (Cl^-, Br^-, SO_4^{2-}, NO_3^- or glutamate), whereas neutral osmolytes have no effect (67).

2.1.4. Specific stimulus

The best-documented case of an osmoregulated system that senses a specific molecule is the membrane-bound sensor kinase KdpD. Consistent with the suggestion of ionic strength as a major stimulus (see previous section), the autophosphorylation activity of KdpD increases up to at least 300 mM NaCl. In the wild-type system, this activity is specifically opposed by K^+ ions, whereas NaCl and KCl stimulate autophosphorylation activity of KdpD to the same extent in a mutant defective in K^+ sensing (51). Unlike the ionic strength signal, K^+ seems to be sensed by KdpD externally (125;140). The stimulation of KdpD activity by ionic strength serves to increase the expression of *kdpFABC* under conditions of osmotic upshift, thereby allowing *E. coli* to counteract the stress by increasing the internal osmolality through the accumulation of K^+ via the KdpFABC ATPase. The inhibitory effect of K^+ is most probably related to the specific function of the KdpD sensor kinase, that is, the induction of the *kdpFABC* operon under hyperosmotic conditions and potassium limitation. This osmoregulatory function is important when the K^+ uptake capacity via the constitutively expressed low affinity systems (TrkG, TrkH and Kup) becomes limiting, *i.e.*, under conditions where the medium concentrations of K^+ are low.

2.1.5. Macromolecular crowding

Cellular volume changes result in changes in cytoplasmic protein concentration (macromolecular crowding), which affect the equilibria of oligomeric enzymes and thereby their function. Although macromolecular crowding will not directly affect the function of systems embedded in the cytoplasmic membrane, membrane proteins that have a tendency to associate with soluble macromolecules may be influenced (37;98). This is an area of research that, in relation to osmotic stress, definitely requires further attention and, in particular, rigorous experimentation.

2.1.6. Physicochemical properties of the membrane

In the context of membrane-protein function, in relation to osmotic stress, the role of bilayer thickness and lateral pressure profile requires

further explanation. Bilayer thickness is obviously important for any membrane protein, as mismatch would result in exposure of hydrophobic surfaces of either the protein or lipid to an aqueous environment. The membrane-water interface of the bilayer comprises a chemically complex environment, which offers multiple possibilities for interactions with protein side-chains (74). If the bilayer thickness is suboptimal for these interactions, the protein or lipid may react to prevent hydrophobic mismatch, which may lead to alterations in protein conformation and activity. Other local intermolecular forces between lipid molecules in a fluid membrane originate from steric hindrance, hydration, electrostatic charge and/or hydrogen bonding in the headgroup region, interfacial tension and acyl-chain pressure. The differences in the nature of the interactions as a function of membrane depth lead to enormous local transverse pressures that correspond to bulk pressures of several hundreds of atmospheres (16). Statistical thermodynamic calculations of the equilibrium pressure profiles of membranes predict large redistributions of lateral pressure when the acyl chain length, the degree, position and configuration of unsaturation, or headgroup repulsion are varied (16). Similarly, the incorporation into a lipid membrane of cholesterol or interfacial active solutes such as anesthetics are predicted to increase the lateral pressure selectively near the aqueous interfaces, resulting in a compensating decrease in lateral pressure near the center of the bilayer. Such changes in the lateral pressure profile have been postulated to influence protein conformation and activity (16).

2.1.7. Mechanic deformation of the lipid bilayer

On the assumption that the lipid bilayer behaves as an elastic solid, the intrinsic mechanical properties of the membrane can be described by four elasticity moduli that describe the response of a unit area of bilayer to volume compression, area expansion, bending/curvature and extension/shear (39). The response of membranes to these elastic deformations has been recently reviewed by Hamill and Martinac (50), and their main conclusions relevant for this paper are summarized here. Firstly, the bilayer is at least 10-fold more compressible in area than in volume during mechanical deformations encountered under physiological conditions. Osmotic downshifts will thus lead to relative increases in membrane area and decreases in membrane thickness. Secondly, the bending rigidity of the bilayer is dependent upon the lipid composition and area of each monolayer, and this parameter determines, amongst others, the shape of lipid vesicles. Thirdly, above the phase transition temperature, the membrane behaves like a fluid in response to extension. Of the elastic deformations, the ones that lead to thinning of the membrane are thought to have major impact on protein conformations and may thus signal activity changes. For the mechanosensitive channel MscL, it is thought that thinning of the membrane upon osmotic downshifts contributes to the ability of the protein to sense membrane tension (50). The tilts of the transmembrane α-helices are predicted to increase as they move away from the axis of the pore, that is, when the membrane expands (141). The osmotic downshift-induced thinning of the membrane could thus provide (part of) the energy required for the transit from the closed to the open state.

2.1.8. Mechanic deformation of proteins

In eukaryotic cells, the cytoskeleton and extracellular matrix affect the function of some membrane transport proteins. For at least one family of eukaryotic mechanosensitive channels, interactions with intra- and extra-cellular proteins are postulated to play an active role in channel gating (148). Traditionally, it was thought always that bacteria lack a cytoskeletal structure but compelling evidence for a bacterial protein that assembles into F-actin like filaments has recently been obtained (61;151). Homologues of this protein, MreB, are widely distributed among rod-shaped,

filamentous and helical bacteria, suggesting that the MreB skeleton is important for the non-spherical shape of these organisms. In addition to this cytoskeleton, most bacteria also have a peptidoglycan exoskeleton or cell wall. This scaffolding could serve a specific role in the regulation of membrane protein function. However, at least for the bacterial mechanosensitive channel MscL and the osmotically regulated transport molecules BetP, OpuA and ProP, no auxiliary protein is required for function. Insertion of pure protein into synthetic lipids is all that is necessary to reconstitute the osmoregulated activity (8)(this thesis)(117;118;129). Similar studies suggest the same is true for YggB, which constitutes the major component of the MscS activity observed in *E. coli* (Moe and Blount, unpublished). On the other hand, a mechanosensitive channel, KefA, which contributes a minor component of the *E. coli* MscS activity, contains a large hydrophilic amino-terminus that is predicted to be periplasmic (84). Much larger than its mechanosensitive counterparts, this channel might have functional interactions with the peptidoglycan layer, or even with outer membrane elements. To date, functional reconstitution has not been reported for this channel. Interestingly, this channel is observed in only about 25% of patches when native membranes of giant protoplasts are assayed by patch-clamp (84); one interpretation is that the channels cluster, perhaps due to cytoskeletal or cell wall interactions. Note that as the radius of curvature increases, less pressure is required to achieve gating tensions (Laplace's law, see above). Hence, localizing these channels to subcellular regions where the membrane assumes a specific radius of curvature (dependent upon where in the *E. coli* rod shaped organism) would effectively set the channels' sensitivity to a specific turgor. Alternatively, auxiliary proteins may be required for gating, or residual cell wall or outer membrane in the minority of patches could explain this phenomenon. In either event, it appears likely that extra-membranous components directly or indirectly regulate the mechanosensitive channel KefA.

Outline of this thesis

As bacterial osmoregulatory systems are key-targets in the control of the growth of pathogens and food spoilage bacteria, it is crucial to develop a good understanding of the osmotic adaptation mechanism(s) of microorganisms. Also from a scientific point of view, it is important to understand the mechanisms controlling cell volume, cytoplasmic osmolality and ionic strength, and protein (de)hydration. This thesis focuses on the identification of the signal underlying the response to an osmotic upshift. The ATP-binding cassette (ABC) transporter OpuA from the Gram-positive lactic acid bacterium *Lactococcus lactis* served as model system for the elucidation of the osmosensing and osmoregulatory mechanism.

In Chapter 2, it is described how an osmotic upshift affects the level of expression and transport activity of OpuA in whole cells.

Chapter 3 describes the over-expression, purification and incorporation of OpuA into liposomes with an ATP-regenerating system. The *in vitro* system was used to study the functional properties of OpuA and to identify the parameters that affect the osmotic activation of membrane-embedded OpuA.

Chapter 4 describes the detailed characterization of the osmosensing mechanism of OpuA, and the identification of the signal that elicits the osmotic activation of OpuA. The parameters possibly affecting the activity of OpuA were varied systematically, as there are, the physicochemical properties of the membrane, and the composition and concentration of the components at the cytoplasmic face of the protein.

In Chapter 5, the directionality of glycine betaine transport and the efficiency of the coupling between ATP-hydrolysis and glycine betaine transport are described.

In Chapter 6, the occurrence of chimeric receptor/translocater ABC-type proteins within the ABC-superfamily is described.

In Chapter 7, the data on OpuA are evaluated in the light of the physiological function of this transporter; some future perspectives are indicated.

Glycine betaine transport in *Lactococcus lactis* is osmotically regulated at the level of expression and translocation activity

Tiemen van der Heide and Bert Poolman

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Summary

Microorganisms react upon hyperosmotic stress by accumulating compatible solutes, whereas the compounds are released upon hypo-osmotic stress. Here we report that *Lactococcus lactis* uses a transport system for glycine betaine that, in *contrast* to earlier observations, is osmotically regulated both at the level of expression and at the level of transport activity. The major causes for the failure to detect osmo-regulated activity are described.

Introduction

In their natural habitats microorganisms are often exposed to changes in the concentration of the solutes in their environment, whereas the internal concentrations of nutrients need to be relatively constant (21;90;110). A sudden increase in the osmolarity of the environment results in the movement of water from the cell to the outside medium, which causes loss of the turgor pressure, changes in intracellular solute concentrations, and cell volume changes. Such hyperosmotic conditions are detrimental to any living cell. Bacteria counteract hyperosmotic stress by accumulating compatible solutes by uptake and/or synthesis. These solutes can be accumulated to high intracellular concentrations without affecting vital cellular processes and they restore the osmotic balance of the cell. Upon hypo-osmotic stress these compatible solutes are released from the cell, which prevents too high a turgor pressure that may ultimately lead to bursting of the cell.

Lactic acid bacteria have limited capacity to synthesize compatible solutes, and a range of studies indicate that glycine betaine, carnitine and proline are the most important compatible solutes in this group of organisms (43;44;100;107;154). The role of these compounds in osmoregulation in other (micro)organisms is also well established (21;45;110;114;122), but in those cases additional molecules play a major role as well. There is a considerable amount of data that indicates that in *Lactobacillus plantarum* and *Listeria monocytogenes* glycine betaine, carnitine and proline are taken up via semi-constitutive transport systems that are activated upon hyperosmotic stress, whereas these compounds are rapidly released by channel-like activities upon osmotic downshock (44;154). These studies prompted us to reinvestigate the

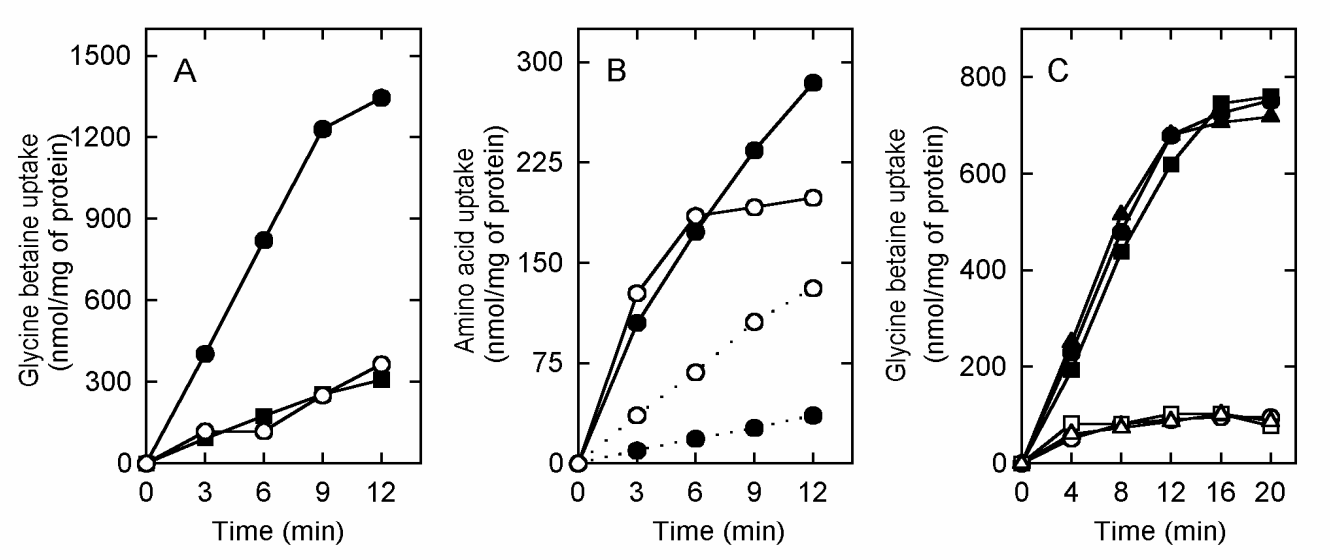

Fig.1. Activation of glycine betaine transport by hyperosmotic conditions. *L. lactis* IL1403 or MG1363 cells were grown semi-anaerobically at 29ºC in CDM (without proline), pH 6.5, plus 25 mM glucose and 500 mM KCl, after which they were washed and resuspended in 50 mM KPi, pH 6.5. (A) Prior to the initiation of transport, IL1403 cells were pre-energized for 5 min with 10 mM glucose. Uptake of [^{14}C]-glycine betaine (1.25 mM, final concentration) was assayed in 50 mM KPi, pH 6.5, with (●,■) or without (○) 500 mM KCl. The reaction was stopped with LiCl at 500 mM (●) or 100 mM (■,○). (B) Effect of hyperosmotic conditions on the uptake of alanine and glutamate. Experimental conditions and symbols are the same as described under A, except that the uptake of [^{14}C]-alanine (solid lines) and [^{14}C]-glutamate (dotted lines) was assayed at 625 µM. (C) Effect of intracellular proline on the uptake of glycine betaine. Prior to the initiation of transport, MG1363 cells were pre-energized for 45 min with 10 mM glucose with (▲,△) or without (●,○) 1.25 mM proline. In a parallel experiment, the proline was added at time zero, that is, simultaneously with the [^{14}C]-glycine betaine (■,□). Uptake of [^{14}C]-glycine betaine was assayed in 50 mM KPi, pH 6.5, containing 50 µg/ml chloramphenicol with (closed symbols) or without (open symbols) 500 mM KCl. The reactions were stopped with 500 mM (closed symbols) or 100 mM (open symbols) LiCl; this was followed by rapid filtration and washing of the filters.

regulation of transport of glycine betaine in *Lactococcus lactis,* which is thought not to respond to any form of osmotic stress whereas the pool sizes during growth in different media do (100). The osmotic regulation of transport through alterations in activity has not only been shown in lactic acid bacteria, but it is also well documented for other bacteria (15;97;110;114). In this communication, we study the regulation of glycine betaine uptake in two well-defined *L. lactis* strains that in many respects are paradigmatic of our knowledge of the physiology, energetics, and genetics of lactic acid bacteria. *L. lactis* MG1363 is a plasmid-free derivative of ML3 that was studied previously (100), whereas IL1403 is a plasmid free strain of which the genome sequence will soon become available.

Regulation of glycine betaine uptake by osmotic upshock

Cells were grown in CDM (with or without proline) (112) plus 25 mM glucose and 500 mM KCl, harvested by centrifugation, and washed and resuspended in 50 mM potassium phosphate, pH 6.5, plus 50 µg/ml chloramphenicol as described (43;44); details are given in the figure legends. The osmotic downshock imposed by the washing step released (most of) the organic compatible solutes from the cell. To initiate the transport reaction, the cells were incubated at 30 ^{o}C in 50 mM potassium phosphate, pH 6.5, plus 10 mM glucose, and after 5 min of preenergization, [^{14}C]-glycine betaine was added to a final concentration of 1.25 mM. To impose hyperosmotic conditions, KCl was added to the

assay medium at a final concentration of 500 mM just prior to the addition of [^{14}C]-glycine betaine. Here, it is important to note that the reactions were stopped by dilution with a 20-fold excess of LiCl solutions that were iso-osmolar with the assay buffer. Fig.1A shows that the uptake of glycine betaine by *L. lactis* IL1403 was stimulated approximately five-fold when the cells were exposed to hyperosmotic conditions during the assay; similar results were obtained with the MG1363 strain. The importance of using iso-osmolar solutions to stop the reaction is evident from the observation that the 'activated' uptake of glycine betaine uptake was no longer observed when the cells were exposed to a hypo-osmotic LiCl (100 instead of 500 mM) solution during the washing step. The fact that washing with a hypo-osmotic solution can obscure osmoregulatory solute transport was also established in other studies (15;97). This is also the reason why in the earlier study by Molenaar *et al.* (100) no osmotic activation of glycine betaine transport was observed.

The uptake of alanine and leucine has previously been shown to be driven by the proton motive force, whereas ATP effects that of glutamate (77). Neither of these transport systems is thought to play a role in osmoregulation. Consistent with this notion, and in contrast to the observations made for glycine betaine, the rate of uptake of alanine, glutamate and leucine was not increased by hyperosmotic conditions (Fig.1B). In fact, the osmotic upshift even inhibited the uptake of glutamate (Fig.1B) and leucine (data not shown).

In *L. plantarum* and *L. monocytogenes,* it was observed that glycine betaine (and carnitine) and proline inhibited the uptake of glycine betaine in *trans;* that is, internal glycine betaine or proline decreased the net rate of uptake (43;154). Glycine betaine and carnitine are taken up in *L. monocytogenes* by separate systems with a high specificity, but both transporters are *trans*-inhibited by glycine betaine as well as carnitine (154). In *L. plantarum*, glycine betaine, carnitine and proline are taken up by one and the same system with an affinity constant for uptake of proline that is almost two-orders of magnitude higher than those for glycine betaine or carnitine (43). The *trans*-inhibition of these systems is largely relieved when the cells are exposed to hyperosmotic stress, thereby permitting the cells to adjust their osmotic imbalance. Fig.1C shows that preloading of *L. lactis* MG1363 with proline under hyperosmotic conditions, which results in proline pools of at least 200 mM (100), did not affect the uptake of glycine betaine; similar results were obtained with the IL1403 strain. This suggests that, in contrast to *L. plantarum*, the uptake of glycine betaine is not *trans*-inhibited by proline within this range. The control experiments show, that under the experimental conditions, the uptake of [^{14}C]-glycine betaine in *L. lactis* was not affected by an equal concentration proline (compare squares and circles), which is consistent with the observation that the system has a much higher affinity for glycine betaine than for proline (100).

To distinguish kinetically between a single or multiple systems and to determine the kinetic effect of osmotic activation, glycine betaine betaine uptake was measured in the range of 0.1 μM to 1.25 mM and in the absence or presence of 500 mM KCl in *L. lactis* IL1403 grown under hyperosmotic conditions. Fig. 2 shows that the maximal rate of uptake (V_{max}) of glycine betaine increased from 19 to 134 nmol min^{-1} mg^{-1} of protein upon osmotic upshock, whereas the affinity constant (K_m) was not significantly affected. The kinetics of glycine betaine uptake was monophasic over the entire concentration range, indicating that a single kinetically distinguishable uptake system is operative. The uptake of glycine betaine is activated by hyperosmotic stress, but the mechanism of osmotic regulation in *L. lactis* seems to be kinetically different from that in *L. plantarum* and *L. monocytogenes*. We conclude that the maximal rate of glycine betaine uptake in *L. lactis* is increased by osmotic upshock through a direct activation of the system, whereas in *L. plantarum* and *L. monocytogenes* the relief of

trans-inhibition forms a major component of the observed activation.

Regulation of expression

The effect of culture conditions on the regulation of expression and activity of the glycine betaine uptake system(s) were studied by cultivating the strains IL1403 and MG1363 in low- and high osmolarity media. The strains were grown in complex (GM17) or synthetic (CDM) media with or without proline and in the presence or absence of 500 mM KCl. Although proline does stimulate the growth of *L. lactis* (138), the amino acid did not have significant effects on the transport activities in the IL1403 strain; some inhibitory effect on transport activity of proline in the growth medium was observed in the MG1363 strain (Table 1). The data clearly indicate that in both strains glycine betaine uptake is induced by high osmolarity, provided the cells are grown in synthetic media. Maximal transport activity was attained when the cells were grown under hyperosmotic conditions and subjected to hyperosmotic stress in the uptake assay. For reasons that are not entirely clear, the effect of hyperosmotic stress on the expression of the glycine betaine uptake system is absent in IL1403 and only moderate in MG1363 when the cells are grown in complex broth (Table 1). Opposite effects such as induction by hyperosmotic conditions and repression by high concentrations of (compatible) solutes, *e.g.* proline or quaternary ammonium compounds present in the complex broth, may be the cause for these observations.

In addition to the use of iso-osmolar buffers to stop the transport reaction in the filtration assay, the experimental conditions here also differed from those of Molenaar *et al.* (100) in a few other aspects. In the previous study, the cells were washed with iso-osmotic buffers and, as a result, the intracellular pool of proline and other compatible solutes were considerable at the start of the uptake assay. Moreover, the effect of an osmotic upshift was only studied in cells grown and washed at low osmolarity, which involved suboptimal expression levels as is shown in Table 1. The present study shows that, in addition to the appropriate assay conditions, the history of the cells in terms of media used for growth and preparation of the cells for the uptake experiments strongly influence observations made with regard to the osmotic regulation of transport.

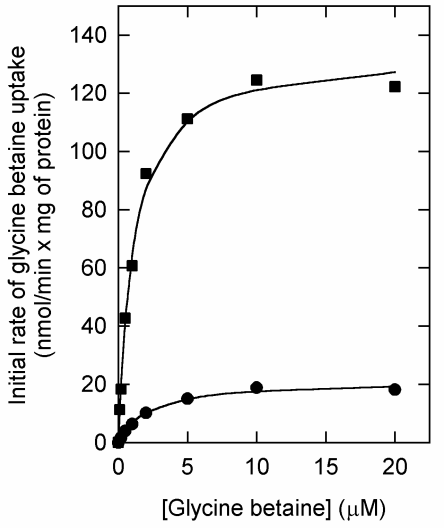

Fig. 2. Kinetic parameters of glycine betaine uptake at hyperosmotic and osmostatic conditions. *L. lactis* IL1403 cells grown in CDM (without proline) plus 25 mM glucose and 500 mM KCl were washed and resuspended in 50 mM KPi, pH 6.5. Prior to the initiation of transport, IL1403 cells were pre-energized for 5 min with 10 mM glucose. Uptake of [^{14}C]-glycine betaine was assayed in 50 mM KPi, pH 6.5, with (■) or without (●) 500 mM KCl. The uptake rates were determined in the concentration range of 0.1 μM to 1.25 mM, but only the data up to 20 μM are shown. The transport assays were stopped after 10 s by diluting the samples 20-fold with ice-cold assay buffer of the corresponding osmolarity; this was followed by rapid filtration and washing of the filters. The data were analyzed with the Michaelis-Menten equation. The K_m values were 1.7 and 1.5 μM in the presence and absence of 500 mM KCl, respectively.

Table 1. Initial rates of glycine betaine uptake in *L. lactis* strains IL1403 and MG1363 grown in media of different osmotic strength and assayed at conditions of low and high osmolarity.

Growth media[a]	Assay buffer (+/- 500 mM KCl)	Activity[b] IL1403	Activity[b] MG1363
CDM –proline	-	< 0.1	5 ± 1
CDM -proline +500 mM KCl	-	3 ± 0.5	20 ± 2
CDM	-	< 0.1	1 ± 0.5
CDM +500 mM KCl	-	1.5 ± 0.5	11 ± 2
CDM –proline	+	11 ± 2	24 ± 2
CDM -proline +500 mM KCl	+	183 ± 30	87 ± 14
CDM	+	18 ± 3	5 ± 0.5
CDM +500 mM KCl	+	131 ± 19	84 ± 9
GM17	-	< 0.1	3 ± 1
GM17 + 500 mM KCl	-	< 0.1	8 ± 2
GM17	+	10 ± 1	10 ± 2
GM17 + 500 mM KCl	+	9 ± 1	52.3 ± 11

[a]CDM contains 675 mg of proline per litre. In the case of GM17, 0.5% glucose was added to the complex broth M17.
[b]Presented are initial rates of glycine betaine uptake in nmol min^{-1} mg^{-1} of protein; the experimental error as estimated from duplicate experiments is indicated.

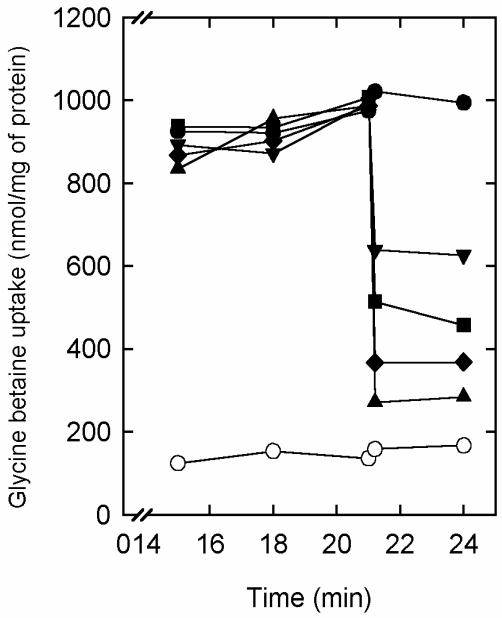

Fig. 3. Glycine betaine efflux is triggered by hypo-osmotic conditions. *L. lactis* IL1403 cells grown in CDM (without proline) plus 25 mM glucose and 500 mM KCl were washed and resuspended in 50 mM KPi, pH 6.5. Prior to the initiation of transport, IL1403 cells were pre-energized for 5 min with 10 mM glucose. Uptake of [^{14}C]-glycine betaine was assayed in 50 mM KPi, pH 6.5, with (●,▼,■,◆,▲) or without (○) 500 mM KCl. After 21 min of uptake the reaction mixtures were not diluted (●,○) or diluted 3- (▼); 5- (■); 10- (◆) or 20-fold (▲) with 50 mM KPi, pH 6.5. The reactions were stopped as described in the legend to Fig.1, using LiCl solutions of the appropriate osmolarity.

Regulation of glycine betaine efflux by osmotic downshock

As suggested by the experiment whose results are shown in Fig.1A, most of the accumulated glycine betaine was released when the cells were washed with hypo-osmotic "stop" solutions. To investigate this efflux in further detail, we diluted glycine betaine-accumulating cells of *L. lactis* to media of various osmolarities (Fig.3). As observed in *L. plantarum* and *L. monocytogenes* (43;44;154), efflux is instantaneous and occurs in proportion to the osmotic downshock. The glycine betaine levels after osmotic downshock were reached within the sampling time of the experiment, that is, a few seconds. The very rapid efflux of glycine betaine is indicative of channel-like activities, as the rates are far too high for catalysis by an 'ordinary' transport system (54;110;143).

Concluding remarks

Glycine betaine uptake in *L. lactis* is subject to osmotic regulation at two levels, that is, at the level of expression and transport activity. In *L. plantarum* the glycine betaine uptake is regulated mainly at the level of transport activity, which involves inhibition by accumulated solute(s) in dependence of the osmotic status of the cells (43). This phenomenon of *trans*-inhibition is not evident from our studies on the osmotic regulation of glycine betaine uptake in *L. lactis*.

Acknowledgements

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Osmoregulated ABC transport system of *Lactococcus lactis* senses water stress via changes in the physical state of the membrane

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Summary

An osmoregulated ABC transporter (OpuA) with novel structural features has been identified that responds to water stress. This glycine betaine transport system consists of an ATP-binding/hydrolyzing subunit (OpuAA) and a protein (OpuABC) that contains both the translocator and the substrate-binding domain. The components of OpuA have been overexpressed, purified, and functionally incorporated into liposomes with an ATP-regenerating system in the vesicle lumen. A *trans*-membrane osmotic gradient (outside hyperosmotic relative to the inside) of both ionic and nonionic compounds was able to osmotically activate OpuA in the proteoliposomal system. Hypoosmotic medium conditions inhibited the basal activity of the system. The data show that OpuAA and OpuABC are sufficient for osmoregulated transport, indicating that OpuA can act both as osmosensor and osmoregulator. Strikingly, OpuA could also be activated by low concentrations of cationic and anionic amphipaths, which interact with the membrane. This result indicates that the activation by a *trans*-membrane osmotic gradient is mediated by changes in membrane properties / protein-lipid interactions.

Introduction

In their natural habitats, microorganisms are often exposed to changes in the concentrations of the solutes in their environment, whereas the internal concentrations of nutrients need to be relatively constant. A sudden increase in the osmolality of the environment results in the movement of water from the cell to the environment, which causes loss of turgor pressure, changes in intracellular solute concentrations, and cell volume changes. Such hyperosmotic conditions are detrimental to any living cell. Osmotic stress can also involve hypo-osmotic conditions, which may cause cell lysis. Bacteria counteract hyperosmotic stress by accumulating compatible solutes by uptake and/or synthesis. This group of solutes includes a whole range of compounds, including amino acids (and analogs), polyols, sugars, quaternary ammonium compounds and potassium, and these compounds can be accumulated to high intracellular concentrations without affecting vital cellular processes. Upon hypo-osmotic stress conditions these compatible solutes are released from the cell. Among the most potent compatible solutes are the quaternary ammonium compounds glycine betaine and carnitine, which are preferentially used by most prokaryotes and eukaryotes (110). The

osmoregulated transporter for quaternary compounds (OpuA) in *L. lactis* constitutes the main system to protect the organism against hyperosmotic stress (52;104), and equivalent activities have been described for other organisms (110;157).

A major question in the field of osmoregulation concerns the mechanism by which the cell senses osmotic stress and transduces the signal(s) to the osmoregulators. The physicochemical parameters that (could) change upon a change in medium osmolality and thus participate in the sensing mechanism include: external water activity, turgor pressure, membrane strain, internal hydrostatic pressure, internal water activity, cytoplasmic volume or concentration of specific signal molecule(s) (21;110;157). The present study eliminates most of these physicochemical factors as signal that is sensed by the glycine betaine transporter of *L. lactis.* In fact, the work indicates that changes in the *trans*-membrane osmotic gradient are transmitted to the OpuA system via distortions in the membrane bilayer, which is consistent with a role for membrane strain (or curvature stress) in the activation mechanism. In order to address the questions pertinent to osmosensing and osmoregulation, the genes encoding the system were cloned and overexpressed, and the proteins were purified and functionally reconstituted into liposomes in which an ATP-regenerating system was entrapped.

Results

ABC transporter with novel structural features

The deduced amino acid sequences of OpuAA (408 amino acids) and OpuABC (573 amino acids), comprising the OpuA system of *L. lactis* IL1403, revealed that the proteins are homologous to OpuAA, OpuAB and OpuAC of *Bacillus subtilis,* but that the domain organization is entirely different. In contrast to the *B. subtilis* system (71), the predicted ligand-binding protein is fused to the C-terminal end of the translocator protein, and the two halves of the binding domain are reversed (104).

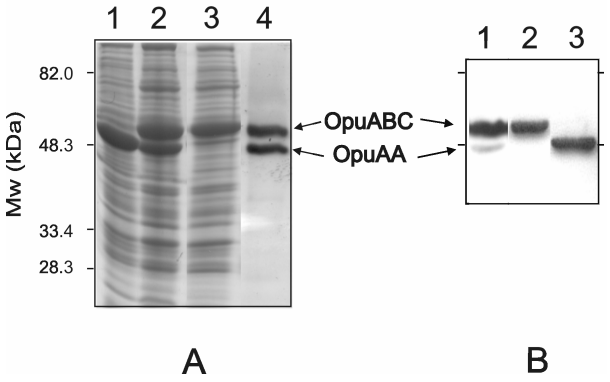

Fig. 1. Amplified expression and purification of OpuA. (A) SDS-PAGE gel (12 % polyacrylamide) showing membranes (Coomasie Brilliant Blue-stained) containing OpuAA (lane 1; 120 µg of protein), OpuA (lane 2; 120 µg of protein), OpuABC (lane3; 120 µg of protein), and purified OpuA (Silver-stained) (lane 4; 15 µg of protein). (B) Immunoblot of membranes with overexpressed OpuA (lane 1; 120 µg protein), OpuABC (lane 2; 120 µg protein), and OpuAA (lane 3; 250 µg protein).

Importantly, the strong sequence conservation between the binding protein(s) (domains) of *L. lactis* and *B. subtilis* suggests that the receptor domain is directly linked to the last trans-membrane segment of the translocator domain. The translocator is predicted to have 8 trans-membrane segments, with both the N-terminus and the C-terminal substrate binding domain located at the extracellular side. There are no indications for an interdomain flexible linker region that is often observed in membrane proteins composed of two or more functional domains (159).

Overexpression, purification and membrane reconstitution of OpuA

Using the nisin inducible expression system (30), both subunits of the OpuA system were amplified up to at least 10 % of total membrane protein. The subunits could be overexpressed separately or in tandem, and cross-reaction with a monoclonal antibody raised against the his-tag confirmed the identity of the components (Fig. 1). The his-tags enabled a one step purification of OpuAA (45 kDa) and OpuABC (62 kDa) using Ni^{2+}-NTA affinity chromatography. Both proteins could also be purified as complex

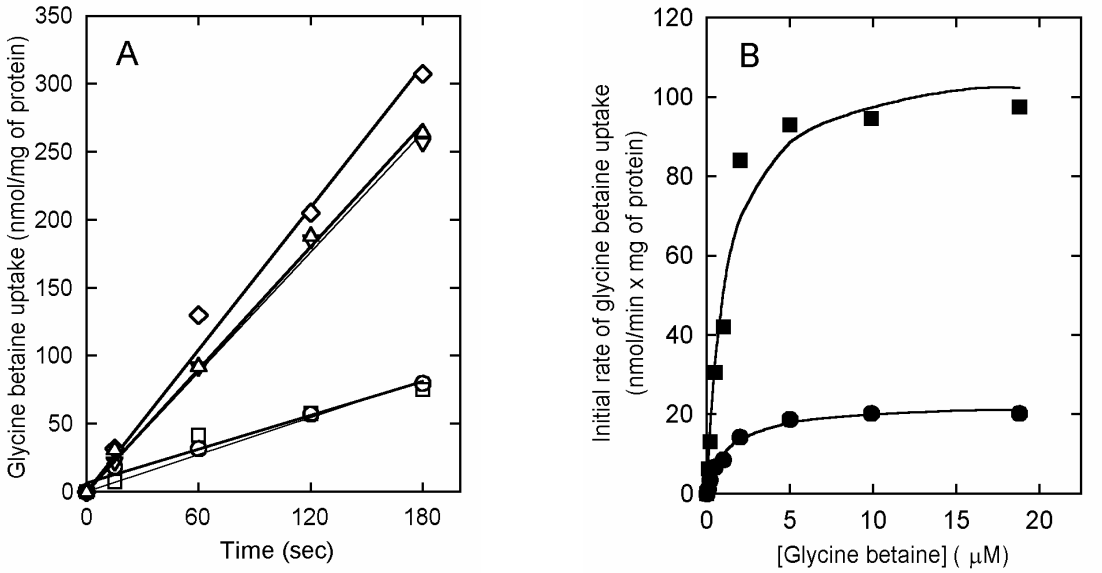

Fig. 2. Osmotic activation of membrane reconstituted OpuA. (A) Uptake of [^{14}C]-glycine betaine (final concentration of 170 μM) was assayed in 90 mM KPi, pH 7.0, under iso-osmotic (○) and hyperosmotic conditions; the latter were effected by the addition of 200 mM KCl (▽), 10.5 % sucrose (◇), 2.5 % glycerol (□), or 2.5 % glycerol + 200 mM KCl (△). The hyperosmotic conditions correspond to 530 mosm/kg for each of the additives. (B) Uptake of [^{14}C]-glycine betaine was assayed in 90 mM KPi, pH 7.0, with (■) or without (●) 200 mM KCl. The uptake was stopped after 15 s and further handling were as described under Experimental procedures. The curves were fitted with the Michaelis-Menten equation.

(OpuA), provided glycerol was present at a concentration of 20 % (v/v) (Fig. 1A; lane 4). The low intensity of the OpuAA band as compared to OpuABC (Fig. 1B; lane 1) is because of a lower blotting efficiency and a reduced sensitivity of the antibody for the 10-histidine tag. Purified OpuA was homogeneous, comprised of equal amounts of OpuAA and OpuABC, and had a purity of more than 95 %. Although n-dodecyl ß-D-maltoside (DDM) was most efficient for solubilization, the purified OpuA complex was reconstituted into Triton X-100-destabilized preformed liposomes as this gave the highest transport activities of (data not shown). To drive the uptake of glycine betaine by OpuA, an ATP-regenerating system, consisting of creatine phosphate, creatine kinase, and ATP, was entrapped in the proteoliposomes. This system assures that the ATP levels remain high and constant, whereas ADP (inhibitor of most ABC-type of transporters) is not accumulated in time. The established uptake rates of glycine betaine were excellent and highly reproducible.

Osmotic activation of OpuA

To address the question if OpuA can act as osmosensor and/or osmoregulator, the system was subjected to hyperosmotic conditions by raising the medium osmolality. A *trans*-membrane osmotic gradient of either ionic (salts) or nonionic compounds (sugars) was able to activate OpuA in the proteoliposomal system (Fig. 2A). From these experiments, we conclude that, besides OpuAA and OpuABC and the lipid membrane, no other cellular components are needed for the functionality, including osmotic activation, of OpuA. The lipophilic compound glycerol, used at comparable osmolality as the other co-solvents, did not activate the system, apparently because it equilibrated across the lipid membrane within the 2 min preincubation period. Importantly, a *trans*-membrane gradient of KCl fully activated OpuA in the presence of glycerol (Fig. 2A). This shows that the OpuA system senses not external or internal osmolality but rather the *trans*-membrane osmotic gradient.

The kinetic parameters for OpuA-mediated glycine betaine uptake in the proteoliposomal system were determined under iso- and

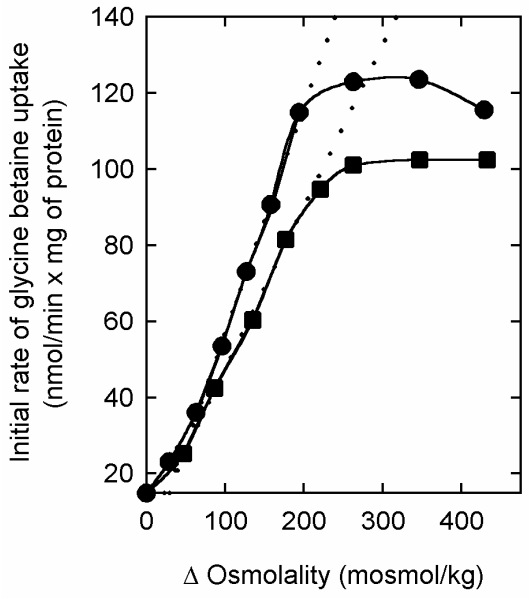

Fig. 3. Threshold value for the *trans*-membrane osmotic gradient needed to activate OpuA. Uptake of [^{14}C]-glycine betaine (final concentration of 80 μM) was assayed in 90 mM KPi, pH 7.0, in the presence of KCl (■) or sucrose (●). Δ Osmolality refers to the difference in external and internal osmolaity.

hyperosmotic conditions (Fig 2B). The apparent maximal rate of glycine betaine uptake (V_{max}) increased from 22.5 ± 0.6 to 108.5 ± 5.0 nmol/min x (mg of protein) when the outside medium was made hyperosmotic. The affinity constant (K_m) for glycine betaine was not affected (1.5 ± 0.3 μM). If ionic and nonionic activate OpuA via the same mechanisms, then the threshold osmolality value of the *trans*-membrane osmotic gradient for KCl and sucrose should be similar. Indeed, Fig. 3 shows that KCl and sucrose activate OpuA at equivalent osmolality. The course of the curve is sigmoid, and the activation threshold for the OpuA system is 20 mosm/kg with a maximal activation reached at 270 mosm/kg. This low threshold osmolality reflects a high sensitivity of OpuA towards hyperosmotic stress. When the outside medium was made hypo-osmotic relative to the inside, the rate of glycine betaine uptake decreased relative to iso-osmotic conditions (data not shown). This shows that the basal activity observed under iso-osmotic conditions represents a state (conformation) of the protein that is in between the inactive and maximally active one.

Effect of amphipathic molecules on OpuA activity

To determine if the OpuA system senses the *trans*-membrane osmotic gradient via changes in the physical properties of the phospholipid membrane, experiments were performed with small amphiphilic molecules that partition in the bilayer. These amphiphiles are predicted to alter the curvature stress in the membrane (16;38), a bilayer property that is also affected by osmotic stress. Fig. 4A shows that low concentrations of the cationic amphipath tetracaine activated the OpuA system under iso-osmotic conditions. The activation was maximal at 1 mM tetracaine. Importantly, these concentrations did not affect the activity of OpuA under hyperosmotic conditions. Concentrations of tetracaine above 1 mM decreased the activation of OpuA both under iso- and hyperosmotic conditions. The amphipath chlorpromazin (cationic) activated OpuA in the same concentration range as did tetracaine (data not shown). The amphipaths did not increase the leakiness of the proteoliposomes for glycine betaine when used at the low concentrations that activate OpuA. Altogether, these results strongly indicate that the sensing of changes in the physical status of the lipid bilayer via lipid/protein interactions provides the trigger for activation of OpuA.

To further assess the effect of tetracaine on OpuA activity, the influence of this amphiphile was tested at different magnitudes of the of the *trans*-membrane osmotic gradient. Although the threshold value for the *trans*-membrane osmotic gradient needed for activation of OpuA was clearly lowered, the addition of tetracaine did not result in an overall shift of the hyperosmotic stress dependence towards lower osmolality values (Fig. 4B).

Discussion

The amplified expression, purification and functional reconstitution of the osmotically activated ABC transport system OpuA is described in this communication. Typical

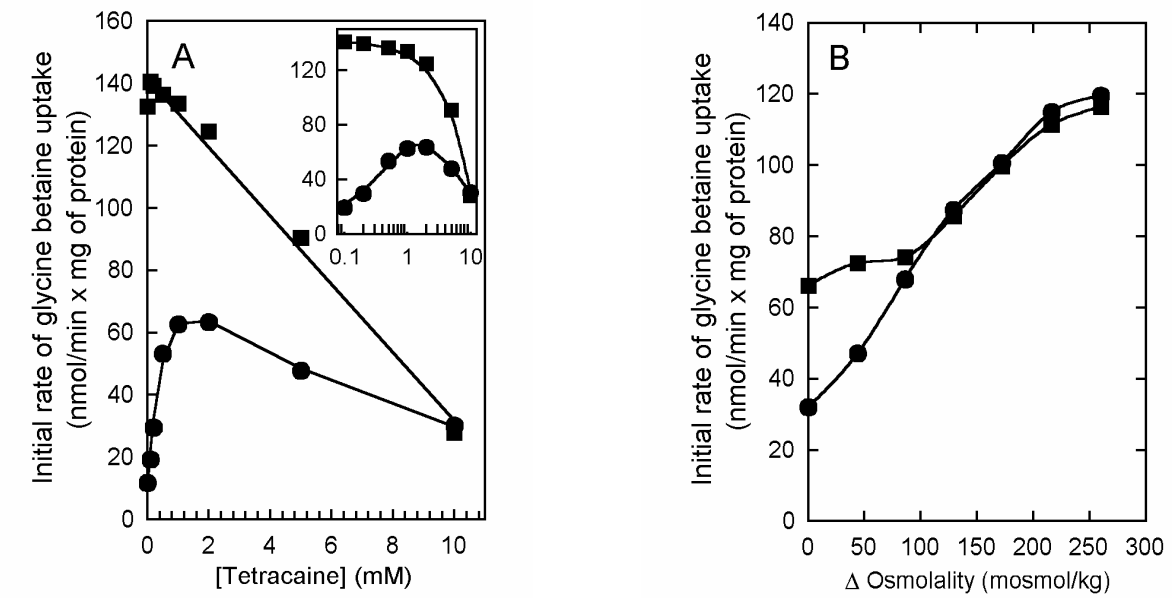

Fig. 4. The effect of tetracaine on OpuA activity. (A) Uptake of [^{14}C]-glycine betaine (final concentration of 80 μM) was assayed in 90 mM KPi, pH 7.0, with (■) or without (●) 400 mM KCl (680 mosm/kg). Both curves were fitted with the double exponential decay function f(x) = a·exp(-b·x) + c·exp(-d·x). The inset shows the data on a logarithmic scale. (B) Uptake of [^{14}C]-glycine betaine (final concentration of 80 μM) was assayed in 90 mM KPi, pH 7.0, with (■) or without (●) 1 mM tetracaine. The osmolality of the medium was varied with KCl. Δ Osmolality refers to the difference in external and internal osmolaity.

prokaryotic binding-protein transporters of the ABC-type are composed of five protein(s) (domains), i.e., an extracellular binding-protein (receptor), two ATP-binding subunits and two integral membrane subunits. Except for the substrate-binding protein, the other subunits can be present as distinct polypeptides or fused to one another, but each entity is always present twice (161). In analogy with other ABC transporters, functional OpuA will most likely be composed of two transmembrane subunits and two ATP-binding subunits. Because OpuABC is composed of a translocator fused to the substrate binding domain, the oligomeric structure implies that two receptor domains are also present. This raises questions about the observations that only a single substrate-binding protein interacts with the dimeric membrane complex, and that two lobes of a single substrate-binding protein interact with different integral membrane protein(s) (domains) (36;87). The oligomeric structure of OpuA of *L. lactis* is not unique, as database searches indicate that similar gene clusters, encoding putative glycine betaine ABC transporters, are present in several families of low-GC Gram-positive bacteria (*Clostridiaceae, Listeriacea, Streptococcaceae*, *Staphylococcaceae)*, high-GC Gram-positive *Actinobacteria* (*Streptomyces*), and the Gram-negative *Helicobacteraceae* within the *ε-Proteobacteria* (104)(3).

Experiments performed in intact cells have shown that hyperosmotic medium conditions stimulate glycine betaine uptake in *L. lactis* through an increased expression and activity of a transport system (52;104). At the onset of this work, the molecular nature of the glycine betaine uptake was unknown, and it was unclear which physicochemical parameter(s) affected the transport activity. We can now rigorously rule out the possibilities that either external or internal water activity per sé, concentration(s) of specific signal molecule(s), compression of the membrane against the cell wall or internal hydrostatic pressure regulate the activity. We conclude that OpuAA and OpuABC are essential and sufficient for the osmotic activation of glycine betaine uptake in *L. lactis*, and that the difference between the external and internal potential of all osmotically active solutes is sensed by the system. This trans-membrane osmotic gradient is sensed via changes in the membrane lipid bilayer as amphiphiles such as tetracaine also trigger the

activation of the transporter. Thus, OpuA not only acts as osmoregulator but also functions as osmosensor for the cell.

The lipid bilayer used for the membrane reconstitution of OpuA constitutes predominantly of phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and phosphatidylcholine (PC), in contrasts to the PG, cardiolipin and glycolipids present in the native membrane of *L. lactis* (35). Given the role of the membrane in transducing osmotic signals to OpuA, it is likely that different physical properties associated with a different lipid composition will affect the transport system. In fact, the higher sensitivity of OpuA towards hyperosmotic stress in the *in vitro* system as compared with the *in vivo* situation may relate to these differences (this work)(52;104). How the headgroup composition, acyl chain lenght, degree of saturation, etc., affect the activity and activation mechanism of OpuA is currently under investigation.

The use of cationic (e.g. tetracaine, chlorpromazine), anionic (e.g. dipyramidole), and neutral amphiphiles in patch-clamp experiments on giant *E. coli* spheroplasts suggested that these compounds potentiate the opening of the mechanosensitive channel MscL by selectively expanding one membrane monolayer and thereby altering the membrane bilayer curvature (93). This is consistent with the gating ('activation') of MscL by hypo-osmotic medium conditions (139;143), as water influx increases the area that is occupied per lipid molecule and thereby alters the curvature stress. OpuA, on the other hand, is activated by hyperosmotic conditions. One would expect that hypo- and hyperosmotic conditions distort the membrane bilayer in a different manner, and that the activation of MscL and OpuA is tuned to these differences. In this regard, it is perhaps surprising that the same amphiphiles that trigger MscL also activate OpuA. The observation that Opu is not only activated by hyperosmotic stress but that the basal activity is decreased by hypo-osmotic conditions is also relevant in this respect. This suggests that osmotic stress acts on OpuA and MscL via a similar mechanism but with a different (osmotic) setpoint for activation, that is, the sign (hypo- versus hyperosmotic) and threshold osmolality difference for activation differs for the two systems. Finally, it is worth emphasizing that the experimental setup for the testing of the effects of the amphipaths on MscL and OpuA differed. MscL has been studied in native membranes (93), in which the composition of the inner and outer leaflet of the membrane bilayer is different. Cationic and anionic amphipath are expected to accumulate in either the inner or outer leaflet, depending on the charge/geometric features of the lipids in the membrane bilayer. In the proteoliposomal system used here, the composition of the two leaflets of the bilayer is by definition the same, and the uneven distribution of tetracaine will be transient as the amphipath flop-flips from the outer to the inner leaflet. At present, it is not known which fraction of the amphipaths flop-flip to the inner leaflet of the membrane on the time scale of the experiments.

Recent theoretical studies suggest that the distribution of the lateral pressure in a lipid bilayer is strongly affected by the incorporation of interfacially active solutes (amphipaths) as well as by an altered lipid composition (16). In these studies, lateral pressure or curvature stress is a function of the depth normal to the protein in the membrane plane, and it is speculated that variations in this stress may be coupled to conformational changes in proteins (16). Future work is aimed at specifying the osmotic activation of OpuA in more detail by correlating threshold values for activation to changes in curvature stress,and identifying the portions in OpuA that actually sense changes in the physical state of the membrane.

While this manuscript was in preparation, a paper was published on the osmotic activation of the sodium-dependent glycine betaine carrier BetP from *Corynebacterium glutamicum* (129). Because there is a fundamental difference in the transport mechanism of the secondary transport system BetP and the ABC transporter OpuA, it will be interesting to find out whether the

osmoregulatory properties of both systems are similar. In contrast to ion-linked transporters such as BetP, OpuA translocates uni-directionally, and only those molecules that are reconstituted in the *in vivo* orientation contribute to the observed uptake. This will facilitate the identification of the region(s) involved in osmosensing when the properties of the inner and outer leaflet of the membrane bilayer are affected differently either through the application of osmotic gradients or specific amphiphaths.

Experimental Procedures

Bacterial strains, growth conditions, and isolation of membrane vesicles

Lactococcus lactis strain NZ9000 (30) was cultivated semi-anaerobically at 30 ºC in M17 broth, pH 6.5, supplemented with 1.0 % (w/v) glucose (GM17 medium) and 5 µg/ml chloramphenicol when carrying pNZopuAhis or derivatives. For the isolation of membranes, cells were grown in a 10 liter pH-regulated fermentor to an OD_{660} of 2, after which transcription from the *nisA* promoter was switched on by the addition of 0.2 % (v/v) culture supernatant of the nisin A producing strain NZ9700 (30). The final concentration of nisin A was about 2 ng/ml. The cells were harvested after one hour of induction and inside-out membrane vesicles were prepared by lysing the bacteria (20 mg/ml) with a French pressure cell (single passage at 10.000 p.s.i.), following (partial) digestion of the cell wall with 10 mg/ml lysozyme for 30 min at 30 °C (113). The membrane preparations were stored in liquid nitrogen.

Plasmid construction

The cloning and sequencing of the *opuA* genes of *Lactococcus lactis* IL1403 will be described elsewhere; the accession number is AF234619**.** For the overexpression of *opuAA* and/or *opuABC,* the genes were placed under the control of the *nisA* promoter of *L. lactis (30)*. The genes were amplified by PCR using the oligonucleotides
AAfor:
5'-GGGCATGCCATGGCAGTAAAAATAAAAATTG-3'
AArev:
5'-GGGCGCGGATCCTTTATTCTCCTCCTC-3';
ABCfor:
5'-GGGCATGCCATGGTTGATTTAGCTATTGGAC-3'
ABCrev:
5'-GGGCGGGGATCCTTTAAACCATTTATC-3'
AAfor plus AArev and ABCfor plus ABCrev were used to amplify *opuAA* and *opuABC* respectively, whereas AAfor plus ABCrev were used to amplify the *opuA* operon. In each case a *Nco*I restriction site was engineered at the start of the (first) gene and a *Bam*HI restriction site at the end of the (last) gene. Following digestion with the corresponding enzymes, the genes were inserted into the *Nco*I/*Bam*HI site of pNZ8048oppAChis (115), thereby replacing the native *oppA* gene; the resulting vectors were named pNZopuAAhis, pNZopuABChis, and pNZopuAhis. Except for *opuAA* in pNZopuAhis, the cloning strategy placed the genes in frame with a sequence specifying a 6-histidine tag at the C-terminus of the protein. In order to engineer a his-tag coding sequence to *opuAA,* pNZopuAhis was digested with *Nco*I/*Xba*I and the DNA fragment was ligated into the corresponding sites of pNZmleP (Bandell M., unpublished). This now placed the *opuA,* the first gene of the *opuA* operon, in frame with a sequence specifying a N-terminal 10-histidine tag. The resulting vector, pNZopuA2his, is otherwise identical to pNZopuAhis and fuses the *opuA* genes translationally to the *nisA* promoter. Finally, the TTG translation initiation codon in *opuAA* was changed into ATG in each of the constructs harbouring this gene.

Purification of OpuA

Membranes were resuspended in buffer A (50 mM KPi, pH 8.0, 200 mM KCl, 20 % glycerol) to a final concentration of 5 mg protein/ml and solubilized with 0.5 % n-dodecyl ß-D-maltoside for 30 min on ice. Following centrifugation, the solubilized material was incubated with Ni^{2+}-NTA resin (0.5 ml of resin/10 mg of membrane protein) for 1 hour at 4 ºC in the presence of 10 mM imidazole. Subsequently, the resin was washed with 20 column volumes of buffer A supplemented 0.05 % Triton X-100 and 15 mM imidazole. The his-tagged proteins were eluted from the column with 3 column volumes of buffer A supplemented with 0.05 % Triton X-100 and 200 mM imidazole.

Immunoblottng

Immunodetection was accomplished using monoclonal antibodies raised against the 6-histidine tag (Dianavo, GmbH). The proteins were separated by SDS-PAGE (12 % polyacrylamide) and transferred to polyvinylidene difluoride membranes by semidry electrophoretic blotting. Detection, by using Western-Light™ chemiluminescence detection kit with CSPD™ as a substrate, was performed as recommended by the manufacturer (Tropix Inc., Bedford, MA)

Membrane reconstitution of OpuA

Liposomes composed of egg phosphatidyl choline (PC) and *Escherichia coli* lipids in a 1:3 ratio were prepared, and membrane reconstitution was performed, essentially as described by Knol *et al*. (76). Briefly, preformed liposomes (4 mg/ml) were destabilized by titration with Triton X-100, and the turbidity of the suspension at 540 nm was used to monitor the physical state of the liposomes. Unless stated otherwise, liposomes destabilized just beyond the point of "detergent-saturation" (75) were mixed with the purified OpuA complex in a 100:1 ratio (w/w), and incubated for 15 min at 4 °C under gentle agitation. To remove the detergent, polysterene beads (Biobeads SM2) were added at a wet weight of 40 mg/ml, and the sample was incubated for another 15 min. Fresh Biobeads SM2 (40 mg/ml) were added to the sample three times and the incubations were continued at 4 °C for 30 min, overnight, and 2 hours, respectively. Finally, the proteoliposomes were collected by centrifugation, washed twice with 50 mM KPi, pH 7.0, and stored in liquid nitrogen.

ATP- energized uptake in proteoliposomes

An ATP-regenerating system, consisting of creatine kinase (2.4 mg/ml), ATP (6 mM), $MgSO_4$ (9 mM), and creatine phosphate (24 mM), was enclosed in the proteoliposomes by two freeze/thaw cycles. Following extrusion of the proteoliposomes through a polycarbonate filter (400 nm pore size), the proteoliposomes were washed twice and resuspended in 90 mM KPi (iso-osmolar with the intraliposomal medium), pH 7.0, to a concentration of 80 mg lipid/ml. Prior to transport, the proteoliposomes were diluted to a lipid concentration of 3.6 mg/ml in 90 mM potassium phosphate, pH 7.0, containing 3 mM $MgSO_4$ (total volume of 220 µl). To impose hyperosmotic conditions, additional salt or sugar was added to the medium. Proteoliposomes were pre-incubated at 30 ºC for 2 min, after which transport was initiated by the addition of radiolabeled substrate. At given time intervals 40 µl samples were taken, and diluted with 2 mL of ice-cold buffer of the same composition and osmolality as the assay medium, except that [^{14}C]/[^{3}H]-glycine betaine was omitted. The samples were filtered rapidly through 0.45 µm pore-size cellulose nitrate filters (Schleicher and Schuell GmbH, Dassel, Germany) and washed once more with 2 ml stop buffer. The radioactivity on the filters was determined by liquid scintillation spectrometry.

Materials

M17 broth was obtained from Difco. Ni^{2+}-NTA resin was obtained from Qiagen Inc., Biobeads SM-2 from BioRad, *n*-dodecyl-β-D-maltoside from Sigma, Triton-X-100 from Boehringer Mannheim. Total *E. coli* lipid extracts and L-α-phosphatidylcholine from egg yolk was obtained from Avanti Polar Lipids. Radiolabeled [N-methyl-^{14}C]- and [N-methyl-^{3}H]-choline-chloride (55 mCi/mmol and 80 Ci/mmol, respectively) were obtained from Amersham (Buckinhamshire, England), and these precursors were used to synthesize [N-methyl-^{14}C]-glycine betaine and [N-methyl-^{3}H]-glycine betaine as described (81). Creatine kinase, creatine phosphate, tetracaine, chlorpromazin, and dipyramidole were obtained from Sigma Chemical Co. All other chemicals were of reagent grade and obtained from commercial sources.

Miscellaneous

The osmolalities of media and buffers were measured by freezing point depression with an Osmostat 030 (Gonotec, Berlin, Germany). The protein concentration was determined by the method of Lowry *et al.* (89), using bovine serum albumin as a standard.

On the osmotic signal and osmosensing mechanism of an ABC transport system for glycine betaine.

Tiemen van der Heide, Marc C.A. Stuart and Bert Poolman

EMBO Journal, **20**, 7022-7032 (2001)
(with some additional information)

Summary

The osmosensing mechanism of the ATP-binding cassette (ABC)-transporter OpuA of *Lactococcus lactis* has been elucidated for the protein reconstituted in liposomes. Activation of OpuA by osmotic upshift was instantaneous and reversible and followed changes in volume and membrane structure of the proteoliposomes. Osmotic activation of OpuA was dependent on the fraction of anionic lipids present in the lipid bilayer. Also cationic and anionic lipophilic amphiphiles shifted the activation profile in a manner indicative of an osmosensing mechanism, in which electrostatic interactions between lipid headgroups and the OpuA protein play a major role. Further support for this notion came from experiments in which ATP-driven uptake and substrate-dependent ATP hydrolysis was measured with varying concentrations of osmolytes at the cytoplasmic face of the protein. Under iso-osmotic conditions the transporter could be activated by high concentrations of ionic osmolytes, whereas neutral ones had no effect, demonstrating that intracellular ionic strength, rather than a specific signaling molecule or water activity, signals osmotic stress to the transporter. The data indicate that OpuA is under the control of a mechanism in which the membrane and ionic strength act in concert to signal osmotic changes.

Introduction

Maintenance of cell turgor is a prerequisite for almost any form of life, as it provides the mechanical force for expansion of the cell wall. Generally, microorganisms respond to an osmotic upshift by accumulating kosmotropic organic solutes (compatible solutes) to counteract the loss of water and decrease in turgor pressure (110). Upon osmotic downshift the cells need to rapidly expel the solutes to prevent turgor pressure from becoming too high, which may lead to lysis of the cells. In order to effectively cope with osmotic stress, the primary response to this type of challenge must involve (in)activation of existing transporters, as synthesis of new enzyme systems would take too long to respond. A major topic in the field of cell volume regulation concerns the mechanism(s) underlying the osmotic activation of transport and channel molecules. The potential stimuli for such systems comprise changes in extra- or intracellular osmolality, ionic strength, turgor pressure, molecular crowding, osmolality gradient across the membrane or the physicochemical properties of the membrane (157). Recent work on the

osmoregulated ATP-binding cassette transporter OpuA from *Lactococcus lactis*, and the ion-linked transporters ProP from *Escherichia coli* and BetP from *Corynebacterium glutamicum* showed that these systems act both as osmosensor and osmoregulator (53;118;129). These studies excluded turgor as possible signal for osmotic activation. Activation of OpuA and BetP by charged amphipaths suggested that these transport systems sense osmotic stress via alterations in membrane properties/protein-lipid interactions, but direct effects via changes in the hydration state of the proteins could not be excluded. Several physicochemical properties of the membrane, such as membrane thickness, fluidity, interfacial polarity, membrane charge, hydration of lipid headgroups, acyl chain packing, but also the 'macroscopic' membrane folding, are affected upon osmolality shifts. Changes in one or more of these membrane parameters, sometimes described in terms of changes in the lateral pressure profile (16), could influence the conformation of the transport proteins and thereby their activity.

Lactococcus lactis responds to an osmotic upshift by accumulating glycine betaine via the ABC transporter OpuA, which is osmotically regulated at the level of both expression and transport activity (52). The protein is composed of two different polypeptides, that is, the ATP-ase and a subunit that comprises both the translocator and ligand-binding domain (53). At present OpuA is one of a few osmoregulated proteins, and the only osmotically activated ABC transporter, that has been characterized in a well-defined proteoliposomal system. Components other than the subunits of OpuA and a lipid membrane are not needed for osmotic regulation of the system. OpuA has the advantage over ion-linked transporters that both translocation and ATPase activity can be followed in a manner that allows discrimination between 'external' and 'cytoplasmic' hydration effects.

In the present study, we show that the osmotic activation profile of OpuA is set by the bulk charge in the lipid headgroup region of the membrane, indicating that electrostatic interactions between lipids and the transporter are intrinsic to the osmosensing mechanism. It is also demonstrated that ionic osmolytes (ionic strength) at the cytoplasmic face of the OpuA protein, rather than a specific molecule or water activity, signals osmotic stress to the transporter. We thus conclude that cytoplasmic ionic strength serves as an osmotic signal, presumably by affecting lipid-protein interactions.

Results

The response of OpuA towards osmotic stress is instantaneous and reversible

To assess the kinetics and reversibility of osmotic (in)activation of OpuA, the membrane-reconstituted protein was alternately exposed to osmotic up- and downshifts. The internal osmolality was varied between 190 and 380 mosmol/kg. Fig. 1 shows that activation of OpuA by osmotic upshift is instantaneous and reversible upon returning to iso-osmotic conditions; the reversibility was not restricted to the particular lipid composition used in this experiment. OpuA was reactivated again by a second osmotic upshift, demonstrating that the integrity of the proteoliposomes was not compromised by the osmotic challenges. Maintenance of liposome integrity was confirmed by internal volume measurements, as shifts between hyper- and iso-osmotic conditions did not result in loss of the enclosed fluorescent dye calcein. It is important to stress here that the internal volume decreased in proportion to the osmotic upshift; the internal and external osmolalities became equal, within the time-resolution of the experiment (~1 sec). Thus, upon osmotic upshift, the surface to volume ratio of the proteoliposome increased.

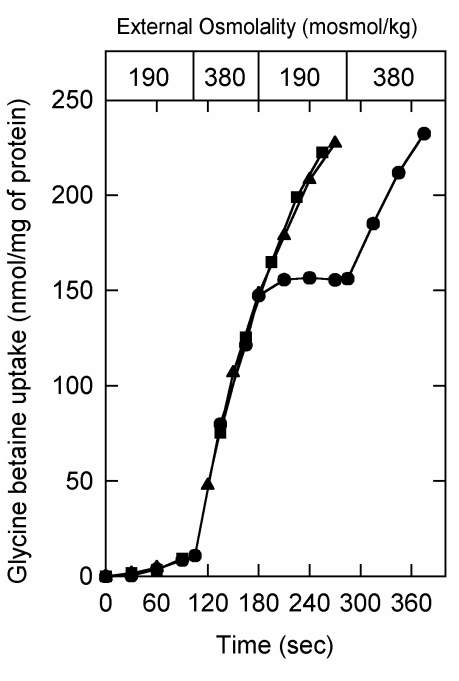

Fig. 1. Kinetics and reversibility of the osmotic activation of OpuA. Uptake of [^{14}C]-glycine betaine (final concentration, 76 µM) was assayed in 100 mM KPi, pH 7.0, corresponding to 190 mosmol/kg. The proteoliposomes were composed of DOPC/DOPE/DOPG in a 2:1:1 mole ratio. At 105 (●,■,▲) and 285 s (●) the proteoliposomes were subjected to hyperosmotic conditions by the addition of 100 mM KCl (final osmolality corresponding to 380 mosmol/kg). Isoosmotic conditions were restored at 180 s (●) by dilution of the assay-mixture with water (plus 76 µM [^{14}C]-glycine betaine).

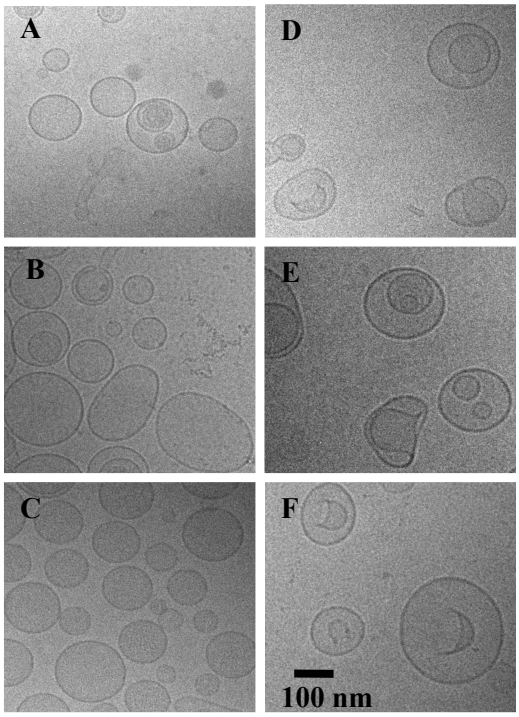

Fig. 2. Morphology of proteoliposomes. Cryo-EM was used to study liposomes composed of mixtures of DOPC, DOPE and DOPG (20 mg/ml, prepared in 100 mM KPi, pH 7.0). Samples were prepared under iso- and hyperosmotic conditions; the latter was effected by the addition of 200 mM KCl. The iso- and hyperosmotic conditions correspond to 190 and 535 mosmol/kg, respectively. (A and D) show liposomes composed of DOPC/DOPE in a 1:1 mole ratio, under iso- and hyperosmotic conditions, respectively. (B and E) show liposomes composed of DOPC/DOPE/DOPG in a 3:4:1 mole ratio, under iso- and hyperosmotic conditions, respectively. (C and F) show liposomes composed of DOPC/DOPE/DOPG in a 1:4:3 mole ratio, under iso- and hyperosmotic, respectively. The vast majority of the proteoliposomes was unilamellar and the appearance of a spherical shape inside a vesicle (panels E and F) generally represents a top view of an invaginated liposome.

Proteoliposomes form 'sickle-shaped' structures when subjected to hyperosmotic stress

To observe the changes in volume of the proteoliposomes upon osmotic upshifts, fluorophore (calcein) self-quenching and cryo-EM studies were performed. It was observed that, at *trans*-membrane osmotic gradients ranging from 0 to 535 mosmol/kg, the volume of the proteoliposomes decreased proportionally to the increase of the osmolality of the medium (data not shown), thereby dissipating the *trans*-membrane osmotic gradient. This osmometric behavior was accompanied by changes in 'macroscopic' folding of the membrane as visualized by cryo-EM (Fig. 2). The proteoliposomes were converted from spherical into 'sickle shaped' structures upon osmotic upshift. In principle, differences in the fraction of anionic lipids could affect the size and 'macroscopic' folding of the membrane, and thereby influence the activity of OpuA. The observed macromolecular structures, however, were the same for all lipid mixtures tested (Fig. 2; data not shown). The proteoliposomes with an average diameter of about 150 nm have a low membrane curvature, but upon osmotic upshift highly curved regions appeared in the proteoliposomes. To exclude the possibility that initial differences in the 'macroscopic'

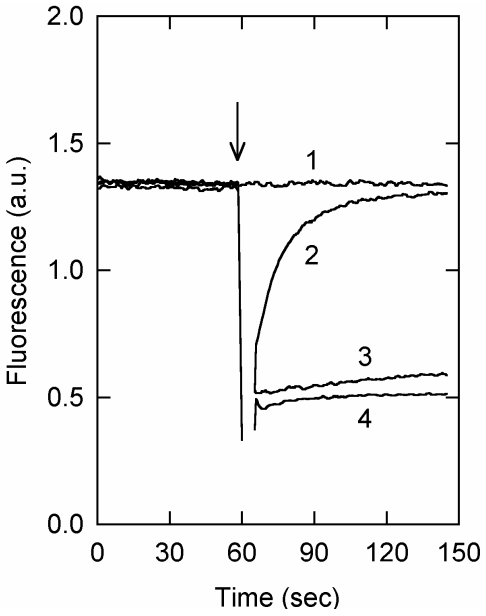

Fig. 3. The effect of membrane-permeable *vs.* membrane-impermeable osmolytes on the internal volume of proteoliposomes. Calcein-quenching was assayed in 100 mM KPi, pH 7.0. The proteoliposomes were composed of 25 mol% DOPC, 50 mol% DOPE and 25 mol% DOPG. Iso-osmotic (1) and hyperosmotic conditions correspond to 190 and 390 mosmol/kg, respectively. To impose hyperosmotic conditions, 200 mM KCl (4), 270 mM glycerol (2) or 327 mM sucrose (3) were added to the sample after 1 min preincubation (indicated by an arrow).

curvature had an effect on the osmotic activation of OpuA, proteoliposomes obtained by extrusion through polycarbonate filters with a pore-size of 200 nm and 400 nm were compared. The experiments showed that the dependence of OpuA on the external osmolality (osmotic activation profile) was the same for both preparations (data not shown).

Membrane-permeable versus membrane-impermeable osmolytes

It has been argued that membrane-impermeable osmolytes such as KCl and sucrose activate osmoregulated transporters by a different mechanism than membrane-permeable osmolytes, e.g. glycerol and low molecular weight PEG's (117). To experimentally test this suggestion, the effects of glycerol on the kinetics of osmotic activation of OpuA and on the changes in volume and macroscopic structure of the proteoliposomes were determined. In the presence of 270 mM glycerol, the amount of glycine betaine taken up after 30 seconds was 30 % higher than that of the iso-osmotic control sample. The stimulation by glycerol was not observed when the osmolyte was added 1 minute prior to the transport measurements (data not shown). Thus, glycerol elicited a transient activation of OpuA that paralleled the time dependence of the shrinkage of the (proteo)liposomes (Fig. 3). The transient changes in liposome volume were confirmed by cryo-EM (data not shown). The kinetics of OpuA activation by glycerol reflects the transient osmotic stress imposed by this highly membrane permeable osmolyte. We conclude that the mechanism underlying osmotic activation of OpuA is irrespective of the type of osmolyte used to challenge the system.

Effect of anionic lipids on the activation profile of OpuA

To investigate if osmotic activation of OpuA is dependent on a particular lipid composition, lipids were used that varied in headgroup size (lamellar vs. non-lamellar lipids), charge (anionic vs. zwitterionic/neutral lipids), acyl chain length (14-22 carbon atoms), and position and configuration of the unsaturated bond in the acyl chain. The predominantly used lipids were phosphatidylglycerol (PG), phosphatidylserine (PS), phosphatidylcholine (PC), and phosphatidylethanolamine (PE).

To determine the specific effects of anionic lipids on OpuA activity, the protein was reconstituted in proteoliposomes composed of the dioleoyl (18:1 Δ9 *cis*) derivatives DOPC (zwitterionic, lamellar), DOPE (zwitterionic, non-lamellar) and DOPG or DOPS (anionic, lamellar) in the mole-ratios 8:8:0, 7:8:1, 6:8:2, 4:8:4, 2:8:6 and 0:8:8, yielding proteoliposomes with 0, 6, 13, 25, 38 and 50 mol% of anionic lipid, respectively. Fig. 4A shows that OpuA was active under iso-osmotic conditions and that an osmotic upshift did not stimulate the activity with 6-13 mol% of anionic lipid present

(only the data for 13 mol% DOPG are shown). OpuA had low or no activity under iso-osmotic conditions when the membrane contained 25 ≥ mol% DOPG. From 25 to 50 mol% anionic lipid, the activation threshold (*trans*-membrane osmotic gradient needed for activation) of OpuA was shifted to higher values. The same trend was observed for the value of the *trans*-membrane osmotic gradient at which maximal activity was reached. The fact that these effects were observed with both DOPG and DOPS (data not shown) suggests that the activation threshold is determined by the fraction of anionic lipids rather than by a specific lipid requirement. No activity was observed in proteoliposomes composed of DOPC or DOPC plus DOPE.

To exclude the possibility that differences in activity were due to variations in reconstitution efficiencies, proteoliposomes consisting of DOPC/DOPE/DOPG (1:2:1 mole ratio) were fused in a one to one ratio with liposomes composed of DOPC/DOPE (1:1 mole ratio), DOPC/DOPE/DOPG (1:2:1 mole ratio) and DOPE/DOPG (1:1 mole ratio), resulting in proteoliposomes containing 50 mol% DOPE plus 13, 25 and 38 mol% DOPG, respectively. These fusion experiments yielded similar activation profiles as the membrane reconstitutions of OpuA in the separate lipid mixtures (Fig. 4B). Fusion of proteoliposomes composed of DOPC or DOPC plus DOPE with DOPG liposomes did not result in significant transport activity despite the fact that all OpuA protein was associated with the vesicles. We conclude that the absence of anionic lipids in the reconstitution process results in trapping of the protein into an inactive state.

Finally, comparable activation profiles of OpuA were observed with ionic (KCl, NaCl, KPi or K_2SO_4) and neutral osmolytes (sucrose) used to vary the external osmolality (data not shown). This indicates that the change in the external ionic strength is not responsible for the observed effects, but that changes in the activation of OpuA are due solely to osmotic effects. It is important to stress here that the majority of the experiments were performed at a relatively high ionic strength (iso-osmotic conditions correspond to 190 mosmol/kg or 100 mM KPi, pH 7.0, externally).

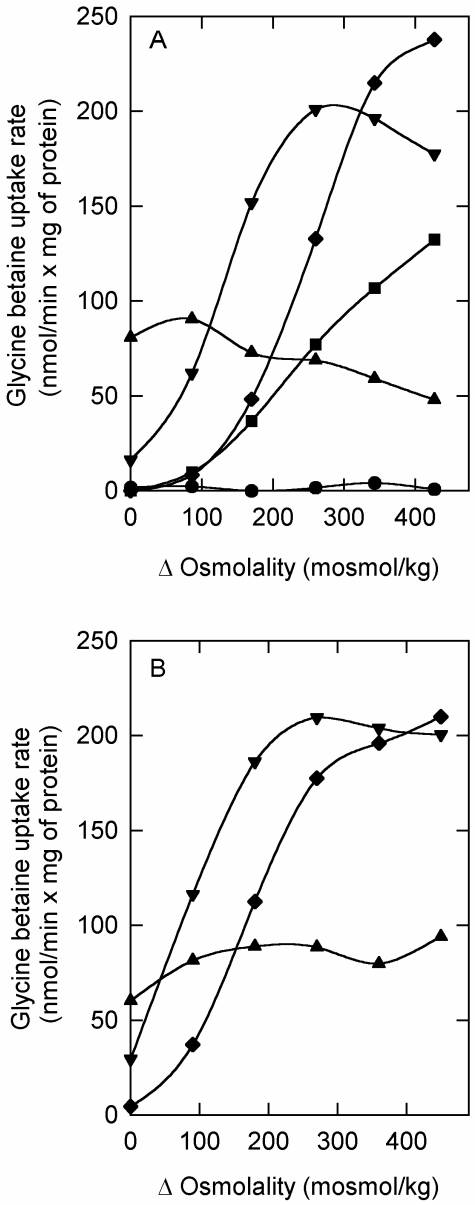

Fig. 4. The effect of anionic lipids on the osmotic activation of OpuA. (A) Uptake of [^{14}C]-glycine betaine (final concentration, 76 μM) was assayed in 100 mM KPi, pH 7.0. The proteoliposomes were composed of 50 mol% DOPE and 0 (●), 13 (▲), 25 (▼), 38 (◆) or 50 mol% DOPG (■) plus 50, 37, 25, 12 or 0 mol% DOPC, respectively. (B) The proteoliposomes were composed of 50 mol% DOPE and 13 (▲), 25 (▼) or 38 mol% DOPG (◆) plus 37, 25 or 12 mol% DOPC, respectively, which were obtained via fusion of proteoliposomes (DOPE/DOPG, 1:1 mole-ratio) with liposomes of the appropriate composition in a 1:1 mole ratio. The osmolality of the medium was varied with KCl. Δ Osmolality refers to the difference in external and internal osmolality.

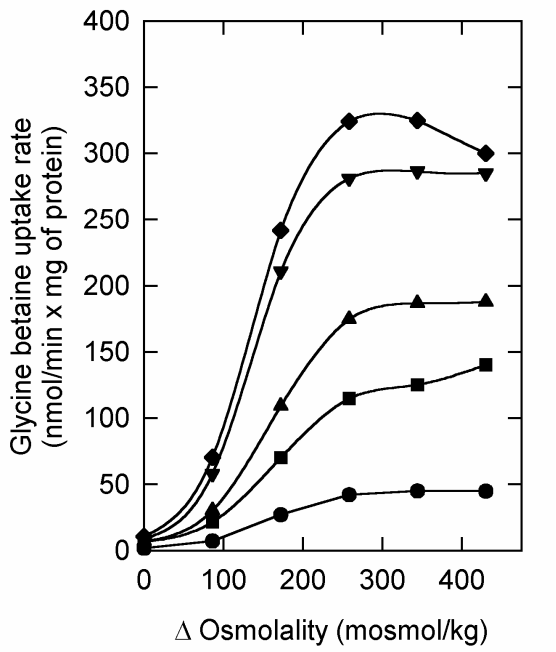

Fig. 5. The effect of non-lamellar lipids (DOPE) on the osmotic activation of OpuA. Uptake of [^{14}C]-glycine betaine (final concentration, 76 µM) was assayed in 100 mM KPi, pH 7.0. The proteoliposomes were composed of 38 mol% DOPG and 6 (●), 13 (■), 25 (▲), 38 (▼), or 50 mol% DOPE (◆) plus 56, 49, 37, 24 or 12 mol% DOPC, respectively, which were obtained via fusion of proteoliposomes (DOPC/DOPE/DOPG, 2:1:1 mole ratio) with liposomes of the appropriate composition in a 1:3 mole ratio. Experimental details are as described in the legend to figure 4.

Effect of non-bilayer lipids on the activation profile of OpuA

To determine the effects of non-bilayer lipids on OpuA activity, the fraction of DOPE was varied between 0 to 50 mol% with DOPG at a constant 38 mol% (Fig. 5). From these experiments it is concluded that DOPE is essential for high activity of OpuA, but the non-bilayer lipid does not affect the activation threshold of OpuA or the value of the *trans*-membrane osmotic gradient at which maximal activity is reached. To further assess the requirement of OpuA for non-bilayer lipids, the effect of one (mono-methyl-DOPE) or two (di-methyl-DOPE) additional methyl groups on the ethanolamine headgroup of DOPE was investigated. The larger the size of the headgroup, the higher the propensity to form stable bilayer structures. It was observed that DOPE, mono-methyl and di-methyl DOPE, in combination with a fixed concentration of DOPG are decreasingly effective in stimulating OpuA activity (data not shown). These observations confirm the notion that non-bilayer forming lipids are needed for maximal activity of OpuA but do not affect the activation mechanism.

Effect of acyl chain length and configuration/position of the unsaturated bond on the activity of OpuA

Besides the headgoup region, the hydrophobic core of the lipid bilayer could influence the activity and/or the osmotic activation profile of OpuA. To determine the effect of the acyl chain length (membrane thickness) on OpuA activity, membrane reconstitution was performed with liposomes consisting of 25 mol% DOPG, 25 mol% DOPE and 50 mol% PC with an acyl chain length of either 14, 16, 18, 20 or 22 carbon atoms (*cis*-unsaturated at the Δ9 position). The activation profiles of OpuA were not affected by the differences in acyl chain length, but the absolute activity reached a maximum in the composition with a PC acyl chain of 18 carbon atoms (data not shown). The osmotic activation profile of OpuA was also not significantly affected when PC (18:1 *trans*-unsaturated at the Δ9 position), PG (18:1 *trans*-unsaturated at the Δ9 position) or PE (18:1 *cis*-unsaturated at the Δ6 position) were used instead of the corresponding dioleoyl (all 18:1 *cis*-unsaturated at the Δ9 position) lipids. Taken together, this dataset indicates that changes in the physicochemical properties of the hydrophobic core of the lipid bilayer are not a determining factor in the osmotic activation mechanism of OpuA.

Effect of amphiphilic molecules on OpuA activity

Since activation of OpuA is strongly dependent on the fraction of anionic lipids present in the membrane, experiments were performed with small charged amphiphilic molecules that partition into the lipid bilayer. Insertion of a cationic or anionic amphipath would lead to a decrease or increase, respectively, of the bulk negative charge at the membrane surface, while neutral amphipaths are predicted to have little effect. With proteoliposomes composed of DOPC/DOPE/DOPG in the mole ratios 2:1:1 and 0:1:1, low concentrations of the cationic amphipath tetracaine in the assay medium indeed decreased the activation threshold of OpuA (Fig. 6A/B; data from experiments with other lipid compositions and/or tetracaine concentrations, not shown). The synergy between ionic strength and the presence of tetracaine was more pronounced at high than low mole fractions of DOPG (e.g. compare Fig. 6A and 6B), suggesting that the effects of tetracaine on OpuA activity may be due not only to perturbation of the membrane surface charge. Figure 6B shows that pre-loading of the proteoliposomes with tetracaine resulted in a more pronounced shift of the activation threshold of OpuA than when the amphipath was added 1 minute prior to the initiation of the transport reaction. This most likely reflects the time needed for tetracaine to equilibrate over the two membrane leaflets. Importantly, and opposite to the effects of tetracaine, the anionic amphipath capric acid (*n*-decanoic acid) shifted the activation threshold of OpuA to higher osmolalities (Fig 6A).

Tetracaine, at a concentration (2 mM) that increased the iso-osmotic activity about 8-fold (Fig. 6A.), did not affect the morphology or surface to volume ratio of the proteoliposomes (data not shown). In these experiments, OpuA is thus not activated via direct changes in water activity, and vesicle shrinkage is not occurring. Since the cationic amphipath tetracaine remains mostly at the phospholipid head-group level, it

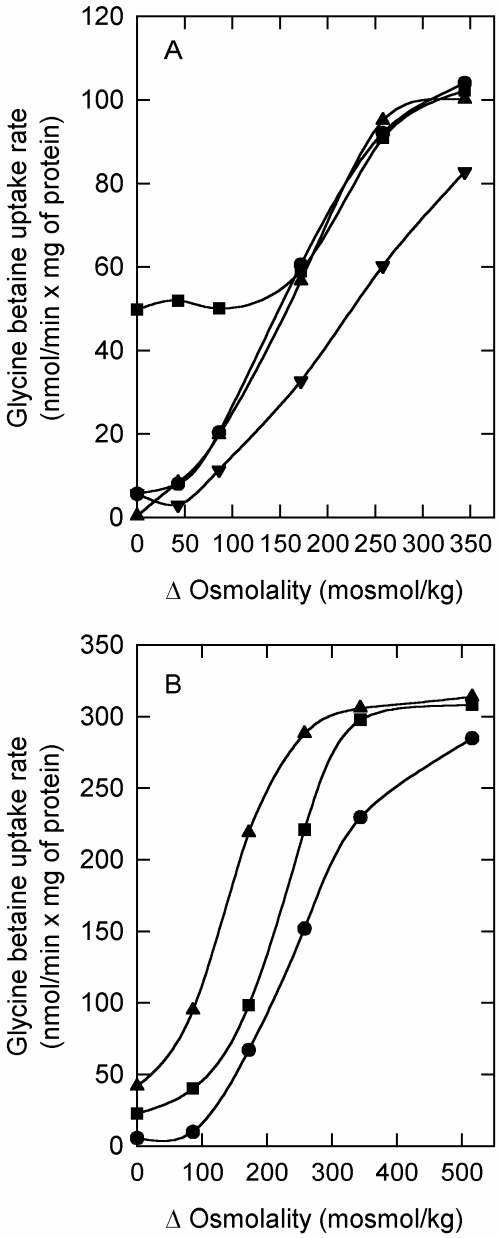

Fig. 6. The effect of membrane-active lipophilic compounds on OpuA activity. Uptake of [^{14}C]-glycine betaine (final concentration, 76 µM) was assayed in 100 mM KPi, pH 7.0. (A) Proteoliposomes were composed of DOPC/DOPE/DOPG in a 2:1:1 mole ratio. Transport activity was assayed without (●) or with tetracaine (■; cationic; 2 mM), decane (▲; neutral; 4.2 mM) or capric acid (▼; anionic; 0.7 mM) present in the assay medium. (B) Proteoliposomes were composed of DOPE/DOPG in a 1:1 mole ratio. Transport activity was assayed in proteoliposomes, non-loaded (●,■) or pre-loaded (▲) with 2 mM tetracaine, in the presence (■,▲) or absence (●) of 2 mM tetracaine in the assay medium. In case of the non-loaded proteoliposomes, the lipophilic compounds were added to the proteoliposomes 1 min prior to the initiation of the transport assay. Experimental details are as described in the legend to figure 4.

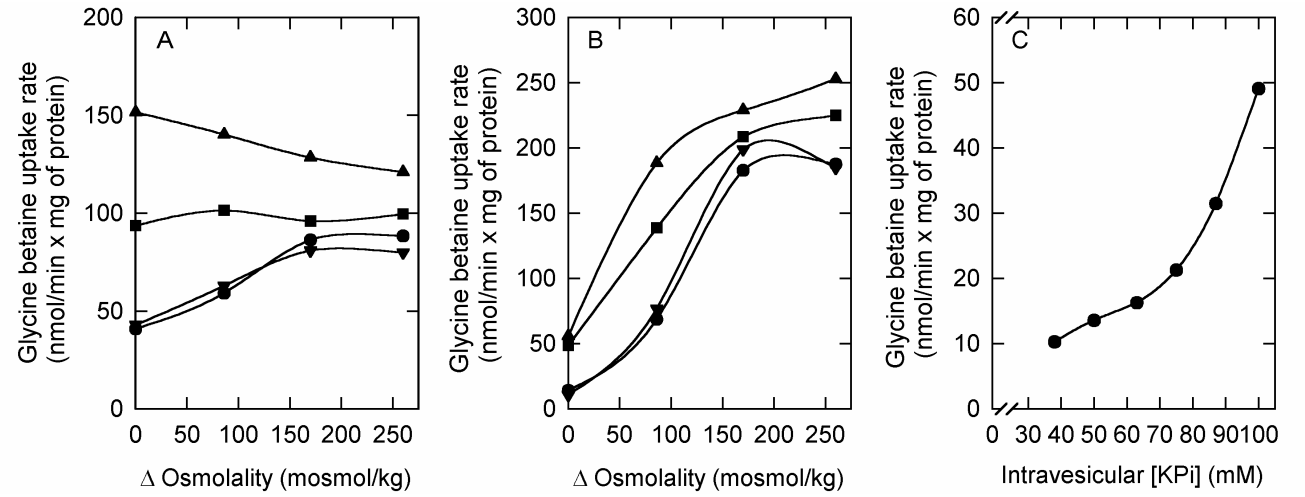

Fig. 7. The effect of ionic strength on the osmotic activation of OpuA. Uptake of [14C]-glycine betaine (final concentration, 76 µM) was assayed in 100 (172 mosmol/kg) or 150 mM (258 mosmol/kg) KPi, pH 7.0. Proteoliposomes were composed of DOPC/DOPE/DOPG in a 58:25:17 (A) and 50:25:25 (B) mole ratio. The standard components (●, 172 mosmol/kg) plus additional KPi (▲), KCl (■) or sucrose (▼) (total of 258 mosmol/kg) were enclosed in the proteoliposomal lumen. (C) Uptake of [^{14}C]glycine betaine (final concentration, 76 µM) was assayed in KPi, pH 7.0 (equi-osmolal to the intravesicular osmolality). Proteoliposomes were composed of DOPC/DOPE/DOPG in a 50:25:25 mole ratio. Potassium phosphate, pH 7.0, was enclosed in the proteoliposomal lumen at the indicated concentrations.

is conceivable that the charged form of the anaesthetic altered the existing intra- and intermolecular electrostatic interactions (11). It is plausible that the same is true for the anionic amphipath *n*-decanoic acid, which shifted the activation profile to higher osmolalities, comparable with an increased fraction of anionic lipids in the membrane. The opposite effects, displayed by cationic and anionic amphipaths, support the view that electrostatic interactions between protein and membrane lipids are intrinsic to the mechanism of osmotic activation. The neutral amphipath lyso-PC and the aliphatic hydrocarbon *n*-decane (Fig. 6A) did not affect the activation profile of OpuA. This suggests that the physical insertion of amphipaths in the lipid bilayer is not sufficient for osmotic activation of OpuA. Additionally, it is known that compounds like tetracaine and *n*-decane increase the membrane fluidity (130;150), which on the basis of our work does not seem critical for osmotic activation of OpuA. Furthermore, the tetracaine-induced activation of glycine betaine transport is paralleled by a corresponding increase of the ATPase activity (data not shown), which indicates that the coupling between transport and ATP hydrolysis is maintained.

High ionic strength at the cytoplasmic face of right-side-out oriented OpuA activates the transporter under iso-osmotic conditions

Since the volume of proteoliposomes decreases upon osmotic upshift, the luminal concentrations of osmolytes increase. If ionic interactions between lipids and protein are intrinsic to the osmosensing mechanism of OpuA, then increases in the intravesicular concentration of ionic osmolytes should affect the activation profile. To discriminate between ionic strength, osmolality and specific osmolyte effects, the intravesicular composition and concentration were varied with the compounds NaCl, KCl, sucrose, fructose, KPi (pH 7.0), NaPi (pH 7.0), K_2SO_4 and Na_2SO_4. Each of these osmolytes was included in the vesicle lumen at a concentration corresponding to 100 mosmol/kg and was present on top of the standard components(50 mM KPi, pH 7.0, plus the ATP-regenerating system), resulting in a total internal osmolality of 290 mosmol/kg. Fig. 7A and B show that each of the ions stimulated OpuA activity, whereas the neutral osmolytes had no effect. Representative data for the osmotic activation profiles of OpuA in proteoliposomes with 17 (Fig. 7A) and 25 mol%

Table I. ATPase activity of membrane-recontituted OpuA[a] as a function of ionic osmolyte concentration.

Osmolytes	Glycine betaine (0.5 mM)	Osmolality (mosmol/kg)	ATPase activities (nmol/min*mg OpuA)
KPi 50 mM	+	90	18 +/- 7 [10%]
KPi 100 mM	+	180	23 +/- 7 [13%]
KPi 150 mM	+	270	117 +/- 16 [65%]
KPi 200 mM	+	360	180 +/- 20 [100%]
KPi 200 mM	-	360	5 +/- 6 [3%]
KPi 250 mM	+	450	146 +/- 15 [81%]
KPi 350 mM	+	630	124 +/- 15 [69%]
KPi (50 mM) + K_2SO_4 (160 mM)	+	450	148 +/- 16 [82%]
KPi (50 mM) + KCl (200 mM)	+	"	99 +/- 12 [55%]
KPi (50 mM) + NaCl (200 mM)	+	"	95 +/- 14 [53%]
KPi (50 mM) + Sucrose (290 mM)	+	"	9 +/- 4 [5%]

[a]Proteoliposomes were composed of 50 mol% DOPC, 25 mol% DOPE and 25 mol% DOPG. The osmolality of the assay medium was varied by the addition of salt or sugar as indicated under osmolytes.

of DOPG (Fig. 7B) are shown. Equi-osmolar concentrations of KPi and K_2SO_4 proved to be more effective in stimulating OpuA activity than the monovalent KCl and NaCl; K^+ and Na^+ were equally effective as cation. To determine the effect of ionic strength on the iso-osmotic activity of OpuA, increasing concentrations of potassium-phosphate were enclosed in the proteoliposomal lumen, thereby varying the internal osmolality from 170 to 290 mosmol/kg. Fig. 7C shows the dependence of the iso-osmotic activity of OpuA on the internal potassium phosphate concentration. A number of conclusions can be drawn from these experiments. First, OpuA seems specifically activated by a high ionic strength in the vesicle lumen, which indicates that OpuA does not sense water activity or a specific signaling. Second, the shifts in the activation profile of OpuA are similar to those when the surface charge of the membrane is altered by varying the fraction of anionic phospholipids or the insertion of charged amphiphiles, which strongly suggests OpuA senses osmotic stress via alterations in the ionic interactions between protein and bilayer lipids.

High ionic strength at the cytoplasmic face of inside-out oriented OpuA activates the transporter

A caveat of the experiments, in which the luminal contents of the proteoliposomes was varied, is that separately prepared proteo-liposome samples were compared. Since a fraction of OpuA is inserted into the proteoliposomes in the 'inside-out orientation', we used this population of molecules to monitor the translocation as a function of external osmolality and ionic strength. The inside-out oriented OpuA molecules affect efflux of glycine betaine when ATP is present on the outside and the appropriate osmotic conditions are imposed. In order to load the proteo-liposomes with glycine betaine, right-side-out oriented OpuA molecules, energized by the ATP-regenerating system inside the vesicle lumen, were used to accumulate the substrate. Fig. 8A shows that the rate of uptake leveled off after a few min; for the cause of this inhibition of uptake, see Chapter 5.

The inside-out oriented OpuA molecules were than supplied with ATP. The inside-out

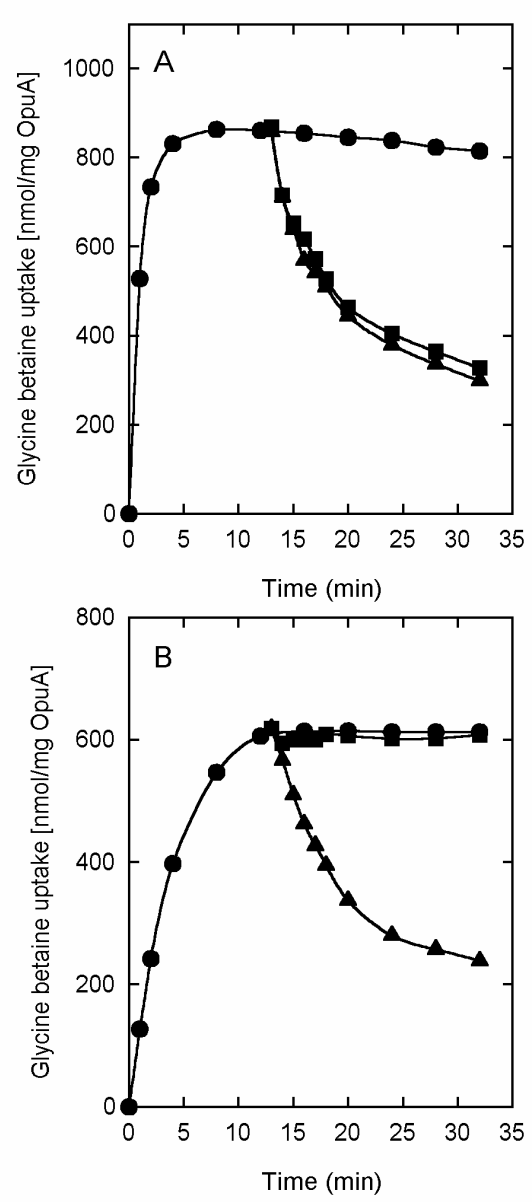

Fig. 8. The effect of ionic vs. nonionic compounds on the osmotic activation of OpuA. Uptake of [^{14}C]-glycine betaine (final concentration, 63 µM,)(M) was assayed in 80 mM KPi, pH 7.0, plus 300 mM KCl (A) or 435 mM sucrose (B). The proteoliposomes were composed of DOPC and DOPE in a 1:1 mole ratio. An ATP-regenerating system was enclosed in the proteoliposomes. After 13 min, ATP and $MgSO_4$ (final concentrations, 9 mM) were added, either together with (▲) or without (■) tetracaine (final concentration, 1.2 mM).

oriented OpuA molecules were only activated when a high enough concentration of ionic osmolytes (KCl or NaC) was present in the outside medium (Fig. 8A; only KCl-data are shown), as the nonionic compound sucrose was unable to activate OpuA (Fig. 8B). In the latter case, however, the inside-out-oriented OpuA molecules could be activated by adding ATP together with a low concentration (1.2 mM) of the cationic amphipath tetracaine. These observations indicate that OpuA is activated by an increase of the ionic strength at the "cytoplasmic" side of the transport protein, which is sensed by membrane-embedded OpuA via changes in the lipid/protein interactions.

High ionic strength at the cytoplasmic face of OpuA increases the ATPase activity

To further characterize the ionic strength dependence of OpuA, glycine betaine dependent ATPase activity was measured. Table I shows that the ATPase activity of OpuA, in proteoliposomes consisting of DOPC/DOPE/DOPG in a 2:1:1 mole ratio, required glycine betaine (in the vesicle lumen with this 'inside-out' system) and a relatively high ionic strength on the outside (the cytoplasmic face of the protein). The ATPase activity increased up to a KPi concentration of 200 mM (Table I). Above 200 mM, KPi became inhibitory in the ATPase assay; a phenomenon also observed when transport activity was measured (data not shown). The activation of the ATPase by ionic osmolytes confirms the observations made with the transport assays, and strongly indicates that *in vivo* osmotic stress is signaled to the protein via alterations in the intracellular ionic strength. A tight coupling between ATPase and transport activity is suggested by the requirement for (internal) glycine betaine when (external) ATPase activity was assayed.

Discussion

Here, we describe the mechanism of osmotic activation of an ABC transporter for glycine betaine. Using a proteoliposomal system with purified protein components and synthetic lipids, and by assaying for ATP-dependent uptake and substrate-dependent ATPase activity, we show that osmotic stress is most probably transduced to the OpuA protein via changes in the intracellular concentrations of ionic osmolytes. The actual osmotic activation profile not only depends on the cytoplasmic

ionic strength but also on the fraction of ionic lipids in the membrane, suggesting that both parameters are interrelated through specific ionic interactions between lipids and protein. Changes in ionic strength are likely to be instantaneous with osmotic (up)shifts, and a mechanism involving (in)activation via changes in the protein-lipid interactions will provide a rapid response to counteract the osmotic imbalance.

With regard to our experimental system, a few aspects are worth emphasizing. First, the osmotic activation of OpuA in proteoliposomes mimics the regulation *in vivo*, which may seem surprising as the vesicles do not withstand turgor, whereas the cell turgor is several atmospheres (157). However, there is increasing evidence that in bacteria, turgor exists only across the cell wall (and outer membrane in case of Gram-negative bacteria) and not across the cytoplasmic membrane (17). This implies that osmosensing devices present in the cytoplasmic membrane cannot respond to changes in turgor, rather, they must sense the consequences of the water influx or efflux, e.g. changes in ionic strength. The consequences of osmotic shifts that OpuA is experiencing in our membrane model system may thus be similar to those in the *in vivo* situation. Secondly, a few other (binding protein-dependent) ABC transport systems have been studied in proteoliposomes, but the coupling between ATPase and translocation activity is generally poor (3;20). Although the details of the coupling between ATP hydrolysis and translocation activity by OpuA will be presented elsewhere, it is important to note that, in experiments such as presented in Table I, ATP hydrolysis is strictly dependent of the presence of substrate. Moreover, the system needs the appropriate 'osmotic stimulus', *i.e.*, high salt when the protein is embedded in a membrane with 25 ≥ mol % of anionic lipids, before ATP hydrolysis takes place. This osmotic stimulus is not required at low concentrations of anionic lipids (e.g. 6 to 13 mol % of DOPG or DOPS). These dependencies match the observations made in the translocation assays.

Kinetics of osmotic activation

In order to cope effectively with osmotic stress, cells need osmotically controllable systems in the membrane at all times, as synthesis takes too long to respond. Here, we show that OpuA is activated instantaneously upon raising the medium osmolality, that is, when the external medium is made hyperosmotic relative to the inside. Activation is elicited by ionic and nonionic osmolytes, provided the molecules do not equilibrate across the membrane on the time scale of the transport measurements (53). Since (proteo)liposomes behave osmometrically, that is, water diffuses across the membrane in response to the osmotic difference between the inner compartment and the outside medium, proteoliposomes are expected to decrease their volume to surface ratio when the outside osmolality is increased. The changes in membrane structure and lumen contents (osmolyte concentration) in osmotically stressed (proteo)liposomes may be compared with those in cells that are in a state of plasmolysis. Proteoliposomes with an average diameter of about 150 nm changed their shape from spherical to sickle-like structures, as shown by the cryo-EM experiments. These morphological changes occurred within milliseconds, *i.e.,* on a time scale much shorter than the interval over which transport was measured. Upon lowering the outside osmolality to the initial value, yielding iso-osmotic conditions again, the vesicles regained their spherical shape and the transporter was deactivated (Fig.1). Thus, osmotic activation and inactivation of OpuA is entirely reversible, occurs on a time scale of seconds or less, and follows the shape and volume changes of the liposomes.

For the proton-linked co-transporter ProP, it has recently been argued that surface hydration of the protein acts as a regulator of ProP activity in addition to osmotic effects that are transduced via the membrane. This suggestion is based on the observation that membrane-permeable osmolytes such as glycerol and low molecular weight PEG's activated ProP to some

extent, whereas these compounds did not affect the volume of the proteoliposomes (117). As membrane-permeant osmolytes rapidly equilibrate across the membrane, one expects the volume decrease as a result of water efflux to be transient as is experimentally shown here. Racher *et al.* (2001) have measured the volume of osmotically challenged liposomes, one minute after the addition of the membrane-permeant osmolyte. At this point of time, the volume of the proteoliposomes had already returned to its initial value when glycerol or low molecular weight PEGs were applied (see Fig.3). Since the activity of ProP was measured within seconds after the addition of permeant osmolytes, rather than after 1 min (kinetic resolution of the volume measurements), the system was in the activated state for a short period of time, resulting in the observed low uptake. Thus, differences in kinetic resolution of the transport and volume measurements may have masked an apparent relationship between the two observations, thereby weakening the argument that ProP activity is regulated via changes in surface hydration of the protein.

Mechanism of osmosensing

Osmotic activation of membrane transport proteins may be triggered through a change in the hydration state of the protein, resulting from the altered water activity (a_w), or the signal may be transmitted to the protein via a specific signaling molecule or a change in the physicochemical properties of the surrounding membrane. Since OpuA is activated not only by osmotic upshift but also by the insertion of cationic amphipaths in the membrane, it seems plausible that the membrane transduces the activation. Osmotic upshift yields sickle-shaped vesicle structures, but these morphological changes are not important *per sé* for the activation of the transporter, as they do not occur upon insertion of amphipaths into the membrane. However, at the molecular level, physical properties such as membrane fluidity, bilayer thickness, hydration state of lipid headgroups, interfacial polarity and charge, and/or lateral pressure may vary with changes in the medium osmolality. To define the osmotic signaling process in more detail, OpuA was incorporated into liposomes of different lipid composition, thereby varying one or more of these parameters. We show that the fraction of anionic (charged) lipids is of major importance for the osmosensing mechanism, whereas variations in acyl chain length, position and configuration (*cis/trans*) of the double bond and fraction of non-bilayer lipids have relatively minor effects. By varying the fraction of anionic lipids (DOPG or DOPS) from <6 to 13, to 25 mol%, OpuA is converted from an inactive (I) to a "constitutively" active (C) to an osmotically controllable (R) state. Moreover, at 25 mol% DOPG, OpuA can be converted from R to C by adding cationic amphipaths, whereas the anionic amphipath decanoic acid shifts the equilibrium towards R. This suggests that the overall charge of the headgroup region of the membrane lipids determines the activity (kinetic state) of the transporter. The possibility that high salt concentrations also interfere with the hydrogen bonding between anionic lipids and OpuA cannot be excluded.

The suggestion that ionic interactions between lipids and OpuA protein determine the activity is supported by the experiments in which the osmolality and osmolyte composition at the cytoplasmic face of the transporter was varied (Fig.7, 8 and Table I). The experiments show that OpuA is activated at iso-osmotic conditions simply by an increase in the concentration of ionic osmolytes. These results confirm that OpuA is not specifically activated by an increase in the concentration of K^+, as both KCl and NaCl suffice as activators and there is no additional regulation via K^+ as proposed for KdpD (68). Recent studies by J. Patzlaff indicate that high concentrations of zwitterions such as carnitine and glycine betaine, presented at the cytoplasmic face of the transporter, also activate the system (unpublished results).

The more efficient stimulation with phosphate and sulfate compared to chloride as anion is consistent with the higher ionic strength

at the same salt concentration. The work also indicates that cytoplasmic ionic strength, rather than internal osmolality or a specific signaling molecule, switches the system between different kinetic states. Because the effects of ionic strength vary with the fraction of anionic lipids in the membrane, and the threshold for osmotic activation is lowered by cationic and raised by anionic amphipaths, the data strongly suggest that the bilayer in which the protein is embedded mediates the osmotic signaling.

Charged amphipaths have previously been used to study osmoregulated systems under conditions that a membrane potential is present across the membrane (93;129), which results in an asymmetric distribution of the molecules over the inner and the outer leaflet of the membrane. In our experimental system, there is no membrane potential and the ultimate distribution will be symmetric. Upon the addition of tetracaine to a liposome suspension, the amphipath will insert into the outer leaflet of the membrane and from there the molecule will flop-flip to the inner leaflet. The shift to lower osmolalities of the osmotic activation profile of OpuA was most pronounced when tetracaine was given the time to equilibrate, i.e., to fill the inner membrane leaflet (Fig. 6B). This is consistent with the idea that the important ionic interactions between protein and lipids reside at the cytoplasmic face of the membrane, allowing internal ionic strength to specifically regulate the activity of OpuA.

Osmotic signal

Our work indicates that a change in intracellular ionic strength serves as a primary signal of osmotic stress for OpuA. We propose that this signal is not sensed by the protein directly, *i.e.,* via changes in surface hydration of the protein or direct effects of the ions on the protein (allosteric site); rather the membrane in which the protein is embedded serves as mediator. Changes in cellular ionic strength are likely to alter specific interactions between (ionic) lipids and the protein, thereby affecting the transport activity. It remains to be established whether the physicochemical parameters of the inner and outer leaflet of the lipid bilayer are equally important for osmotic activation of OpuA. The effects of ionic strength, however, are exerted only at the cytoplamic face of the protein.

Concluding remarks

How general are the observations and concepts reported here for the osmoregulated ABC transporter OpuA? Well-studied transporters with a similar function, but structurally unrelated to OpuA, are the ion-linked transporters ProP from *E. coli* (or *Staphylococcus typhimurium*) and BetP from *Corynebacterium glutamicum* (118;129). Similar to the observations made for OpuA, the optimum of osmotic stimulation of BetP from *C. glutamicum* shifts to higher values of osmolality with increasing amounts of phosphatidylglycerol lipids in the membrane, pointing towards a role of the membrane in transducing ionic changes to the protein (129). We have already argued that one does not need to invoke surface hydration of ProP as regulatory mechanism (and thus internal osmolality as a signal) to explain the observations. Moreover, *in vivo* studies on this protein have indicated that activation of ProP requires the presence of K^+ in the medium (78). Since K^+ is rapidly accumulated in the initial response of *E. coli* to hyperosmotic stress, the requirement for K^+ is consistent with a role for intracellular ionic strength in the regulation of ProP, but other factors such as an increase in the intracellular pH may have contributed as well (110).

Also for the osmotic regulation of gene expression there is evidence that intracellular ionic strength serves as a signal (56). Thus, altogether there are clear indications that nature has used similar principles for the evolvement of different types of osmoregulatory circuits.

Experimental Procedures

Bacterial strains, growth conditions, and isolation of membrane vesicles

Lactococcus lactis strain NZ9000 (30) was cultivated semi-anaerobically at 30 ºC in M17 broth, pH 6.5, supplemented with 1.0 % (w/v) glucose (GM17 medium) and 5 µg/ml chloramphenicol when carrying pNZopuAhis or derivatives. For the isolation of membranes, cells were grown in a 10 liter pH-regulated fermentor to an OD_{660} of 2, after which transcription from the *nisA* promoter was switched on by the addition of 0.2 % (v/v) culture supernatant of the nisin A producing strain NZ9700 (30). The final concentration of nisin A was about 2 ng/ml. The cells were harvested after one hour of induction and inside-out membrane vesicles were prepared by lysing the bacteria (20 mg/ml) with a high pressure homogenizer (Kindler type NN2002; single passage at 10000 p.s.i.), following (partial) digestion of the cell wall with 10 mg/ml lysozyme for 30 min at 30 °C (113). The membrane preparations were stored in liquid nitrogen.

Purification of OpuA

Membranes were resuspended in buffer A (50 mM KPi, pH 8.0, 200 mM KCl, 20 % glycerol) to a final concentration of 5 mg protein/ml and solubilized with 0.5 % n-dodecyl ß-D-maltoside (DDM) for 30 min on ice. Following centrifugation, the solubilized material was incubated with Ni^{2+}-NTA resin (0.5 ml of resin/10 mg of membrane protein) for 2 hours at 4 ºC in the presence of 15 mM imidazole. Subsequently, the resin was washed with 20 column volumes of buffer A supplemented 0.05 % Triton X-100 and 15 mM imidazole. The his-tagged proteins were eluted from the column with 3 column volumes of buffer A supplemented with 0.05 % Triton X-100 and 200 mM imidazole.

Membrane reconstitution of OpuA

Liposomes composed of the desired lipids were prepared, and membrane reconstitution was performed, essentially as described by Knol *et al.* (76). Briefly, preformed liposomes (4 mg/ml) were destabilized by titration with Triton X-100, and the turbidity of the suspension at 540 nm was used to monitor the physical state of the liposomes. Unless stated otherwise, liposomes destabilized to a point just beyond "detergent-saturation" (75) were mixed with purified OpuA in a 100:1 ratio (w/w), and incubated for 30 min at room temperature (RT) under gentle agitation. To remove the detergent, polysterene beads (Biobeads SM2) were added at a wet weight of 40 mg/ml, and the sample was incubated for another 15 min. Fresh Biobeads SM2 (40 mg/ml) were added to the sample four times and the incubations were continued at 4 °C for 15 min, 30 min, overnight, and 2 hours, respectively. Finally, the proteoliposomes were collected by centrifugation, washed twice with 50 mM KPi, pH 7.0, and stored in liquid nitrogen.

ATP- driven uptake in proteoliposomes

An ATP-regenerating system, consisting of creatine kinase (2.4 mg/ml), ATP (6 mM), $MgSO_4$ (9 mM), and creatine phosphate (24 mM), was enclosed in the proteoliposomes by three freeze/thaw cycles. Following extrusion of the proteoliposomes through a polycarbonate filter (200 nm pore size), the proteoliposomes were washed twice and resuspended in 100 mM KPi (iso-osmolar with the intraliposomal medium), pH 7.0, to a concentration of 80 mg lipid/ml. Prior to transport, the proteoliposomes were diluted to a lipid concentration of 3.6 mg/ml in 100 mM potassium phosphate, pH 7.0 (total volume 50 µl). To impose hyperosmotic conditions, additional salt or sugar was added to the medium. Unless stated otherwise, proteoliposomes were pre-incubated at 30 ºC for 1 min, after which transport was initiated by the addition of radiolabeled substrate. After given time intervals the samples were diluted with 2 ml of ice-cold buffer of the same composition and osmolality as the assay medium, except that [^{14}C]-glycine betaine was omitted. The samples were filtered rapidly through 0.45 µm pore-size cellulose nitrate filters (Schleicher and Schuell GmbH, Dassel, Germany) and washed once more with 2 ml stop buffer. The radioactivity on the filters was determined by liquid scintillation spectrometry. Initial rates of transport were obtained from the linear increase in glycine betaine uptake over the first 30 seconds, and triplicate samples were taken for analysis. Each experiment was repeated at least twice.

Cryo-electron microsopy

A small droplet of phospholipid vesicle suspension (20 mg/ml) was placed on a glow discharged holey carbon coated grid. The excess of liquid was blotted away with a filter paper and the grid was vitrified in liquid ethane. To avoid evaporation during the procedure, the grid was placed in a closed chamber over-saturated with water. This was achieved by using an ultrasonic vaporizer, which creates a cloud around the specimen (Frederik *et al.*, in preparation). The grids were examined in a Philips (Eindhoven, The Netherlands) CM120 cryo-electron microscope operating at 120 kV equipped with a Gatan cryo-stage (model 626). Images were recorded under low-dose conditions with a Gatan (model 794) slow-scan CCD camera.

Internal volume measurements

The fluorophore calcein was prepared at a concentration of 100 mM in a 50 mM potassium phosphate and the pH was adjusted to 7.0 by using KOH. Calcein (final

concentration: 10 mM calcein in 85 mM KPi, pH 7.0, corresponding to 190 mosmol/kg) was enclosed in the (proteo)liposomes by three freeze/thaw cycles. Following extrusion of the (proteo)liposomes through a polycarbonate filter (200 nm pore size), the external calcein was removed by applying the sample to a NAP-10 column (Pharmacia), after which the (proteo)liposomes were eluted, washed twice and resuspended in 100 mM KPi, pH 7.0 (190 mosmol/kg). A calibration curve for calcein-quenching was made by preparing a range of (proteo)liposomes in which calcein was enclosed at different concentrations but keeping the internal osmolality constant at 190 mosmol/kg. Fluorescence measurements were made with an Aminco SPF-500 spectrofluorometer at an exitation wavelength of 495 nm and an emission wavelength of 520 nm (with slit widths of 10 nm). The fluorescence measurements were started by diluting 4 µl of calcein-loaded (proteo)liposomes (20 mg/ml lipid) into 1 ml of 100 mM KPi, pH 7.0. To impose hyperosmotic conditions, additional osmolytes were added to the sample after 1 minute pre-incubation. Samples were stirred and the fluorescence measurements were performed at 25ºC. The maximal unquenched calcein fluorescence-signal was determined by solubilizing the liposomes with 0.1 % of Triton X-100.

ATPase assay

Proteoliposomes were used in which OpuA was reconstituted in a 1:50 ratio (w/w). The proteoliposomes were frozen and thawed two times with or without glycine betaine (1 mM) present. Following extrusion of the proteoliposomes through a polycarbonate filter (200 nm pore size), the proteoliposomes were washed twice and resuspended in 50 mM KPi (iso-osmolar with the intraliposomal medium), pH 7.0, to a concentration of 100 mg lipid/ml. The assay was initiated by diluting the liposomes two-fold with an iso-osmotic phosphate buffer containing ATP (6 mM) and $MgSO_4$ (6 mM). To impose hyperosmotic conditions, additional salt or sugar was added to the medium. At given time intervals, samples were drawn and diluted 500-fold with 50 mM KPi, pH 7.0, and assayed for ATP by using the ATPLite^{TM}-M system (Packard).

Materials

M17 broth was obtained from Difco. Ni^{2+}-NTA resin was obtained from Qiagen Inc., Biobeads SM-2 from BioRad, *n*-dodecyl-β-D-maltoside from Sigma, Triton-X-100 from Boehringer Mannheim. Total *E. coli* lipid extracts, L-α-phosphatidylcholine from egg yolk and synthetic lipids were obtained from Avanti Polar Lipids. Radiolabeled [N-methyl-^{14}C] and [N-methyl-^{3}H] choline-chloride (55 mCi/mmol and 80 Ci/mmol, respectively) were obtained from Amersham (Buckinhamshire, England), and these precursors were used to synthesize [N-methyl-^{14}C]-glycine betaine and [N-methyl-^{3}H]-glycine betaine as described (81). Creatine kinase, creatine phosphate, and tetracaine were obtained from Sigma Chemical Co. All other chemicals were of reagent grade and obtained from commercial sources.

Miscellaneous

The osmolalities of media and buffers were measured by freezing point depression with an Osmostat 030 (Gonotec, Berlin, Germany). The protein concentration was determined by the method of Lowry *et al.* (89), using bovine serum albumin as a standard.

Acknowledgements

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Glycine betaine transport by the ABC transporter OpuA is unidirectional and tightly coupled to ATP-hydrolysis.

Tiemen van der Heide and Bert Poolman

Summary

Microorganisms react to an osmotic upshift by accumulating compatible solutes. Here, we report that the uptake of glycine betaine via the osmotic-upshift activated ABC transporter OpuA from *Lactococcus lactis* is unidirectional and strictly coupled to ATP-hydrolysis, with a stoichiometry of 2 to 4.

Introduction

In ABC transporters, the hydrolysis of ATP is stoichiometric with the transport of substrate. Secondary transporters, on the other hand, utilize the free energy difference of the electrochemical gradients of the translocated species across the membrane. The large free energy difference associated with the hydrolysis of ATP allows the accumulation of solutes to much higher levels than possible with conventional secondary transport mechanisms. The fact that these systems operate far from thermodynamic equilibrium has led to the widespread acceptance that ABC transporters catalyze a unidirectional transport. However, for three amino acid ABC transporters, the general and branched-chain amino acid permeases from *Rhizobium legiminosarum*, and the histidine permease from *Salmonella. typhimurium,* it has been reported that besides uptake the transporters also mediate efflux of substrate (58). Given the conservation of structure and mechanism of ABC transport systems, it has been proposed that bi-directional solute movement might be a general feature of this superfamily (58).

A consequence of the proposed bidirectional mechanism is that a system would not be able to accumulate substrates to high levels, not more than 4 mM in case of the *R. legiminosarum* glutamate ABC transporters (58). The directionality of glycine betaine transport of the osmoregulated ABC transporter OpuA from *Lactococcus lactis* is presented here. This transporter is involved in the response of the organism to an osmotic upshift (increase in the extracellular osmolality), thereby counteracting the loss of water and the accompanying drop in turgor (52). This is achieved by the rapid uptake of glycine betaine, a compatible solute that can be accumulated to (sub-)molar levels in the cytoplasm without disturbing vital cellular processes. Previous studies in an *in vitro* membrane-model system showed that OpuA is activated when subjected to an osmotic upshift, with the increase in ionic osmolyte concentration at the cytoplasmic face of the system acting as the primary signal for activation (54). Changes in the lipid/protein interactions seem to mediate the osmosensing and activation, indicating that the membrane and ionic osmolytes act in concert to signal osmotic changes. This notion was supported by the observation that small charged amphipathic

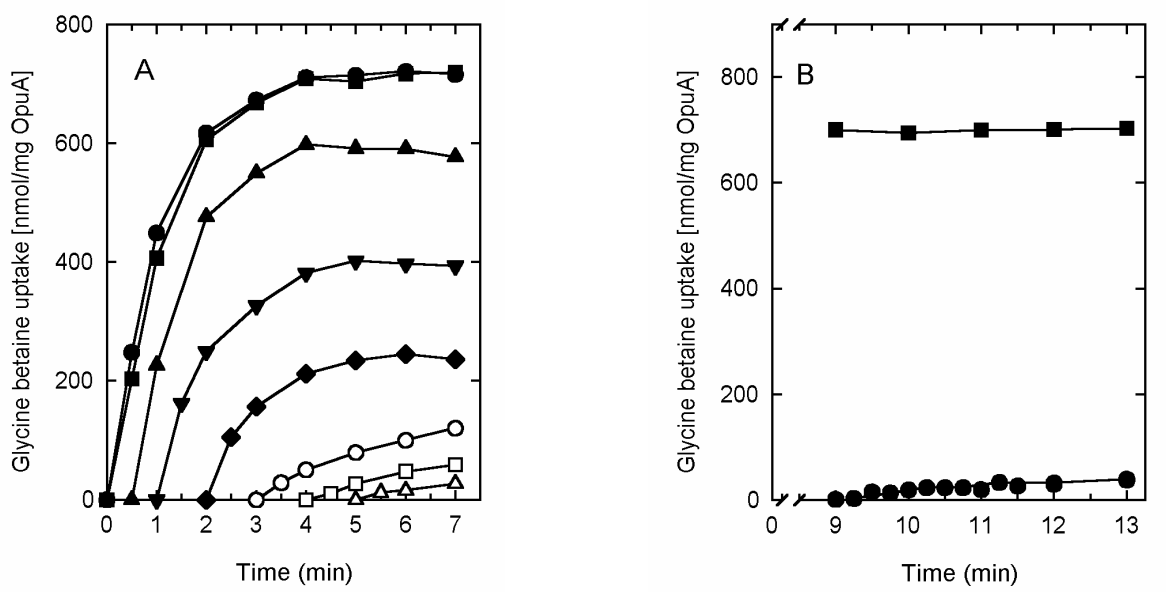

Fig. 1. Directionality of glycine betaine transport by OpuA. A) Uptake of [^{14}C]-glycine betaine (final concentration, 63 µM,)(●) was assayed in 80 mM KPi, pH 7.0, plus 300 mM KCl. An ATP-regenerating system was enclosed in the proteoliposomes. Tracer amounts of [^{3}H]-glycine betaine (final concentration, 45 nM) were added to separate transport assays at 0 (■) and after 0.5 (▲), 1 (▼), 2 (◆), 3 (○), 4 (□) and 5 (△) min. B) Uptake of [^{14}C]-glycine betaine (final concentration, 63 µM,)(■) was assayed in 80 mM KPi, pH 7.0, plus 300 mM KCl. Tracer amounts of [^{3}H]-glycine betaine (final concentration, 45 nM) were added after 9 min (●).

molecules, interacting with the membrane, could mimic the effect of an osmotic upshift (53).

In the present work, it is shown that the transport of glycine betaine by OpuA is unidirectional and strictly coupled to the hydrolysis of ATP. Estimates of the transport reaction-stoichiometry are presented, that is, the number of ATP molecules hydrolyzed per substrate molecule transported.

RESULTS

OpuA operates unidirectionally

When membrane-reconstituted OpuA is subjected to an osmotic upshift, it is triggered to rapidly accumulate glycine betaine, but after some time the transport rate levels off. This reduction in transport rate could be due to depletion of ATP, build up of ADP or glycine betaine efflux that balances the uptake. To determine whether passive efflux or efflux mediated by OpuA causes the reduction in the apparent uptake rate, two different labels ([^{14}C]- and [^{3}H]-glycine betaine) were used to follow the transport reaction. Proteoliposomes were allowed to transport [^{14}C]-glycine betaine and this label was used to monitor the overall uptake. Tracer amounts of [^{3}H]-glycine betaine were added at successive time points to determine the actual rate of uptake at times that the proteoliposomes had pre-accumulated glycine betaine to varying extent. When the transport reaction is unidirectional, it is expected that the deduced transport rates of [^{3}H]- and [^{14}C]-glycine betaine are similar in the course of the experiment, whereas the deduced rate of [^{3}H]-glycine betaine accumulation is expected to be higher than that of [^{14}C]-glycine betaine when pre-accumulated [^{14}C]-glycine betaine leaks out or inhibits further uptake. The results clearly show that glycine betaine uptake is unidirectional and not inhibited by pre-accumulated glycine betaine, as the transport rates of [^{3}H]-glycine betaine and [^{14}C]-glycine betaine are identical throughout the course of the transport reaction (Fig. 1). This suggests that ATP-depletion and/or the build up of ADP, a known inhibitor of ABC transporters, brings about the observed *steady state* level of glycine betaine accumulation.

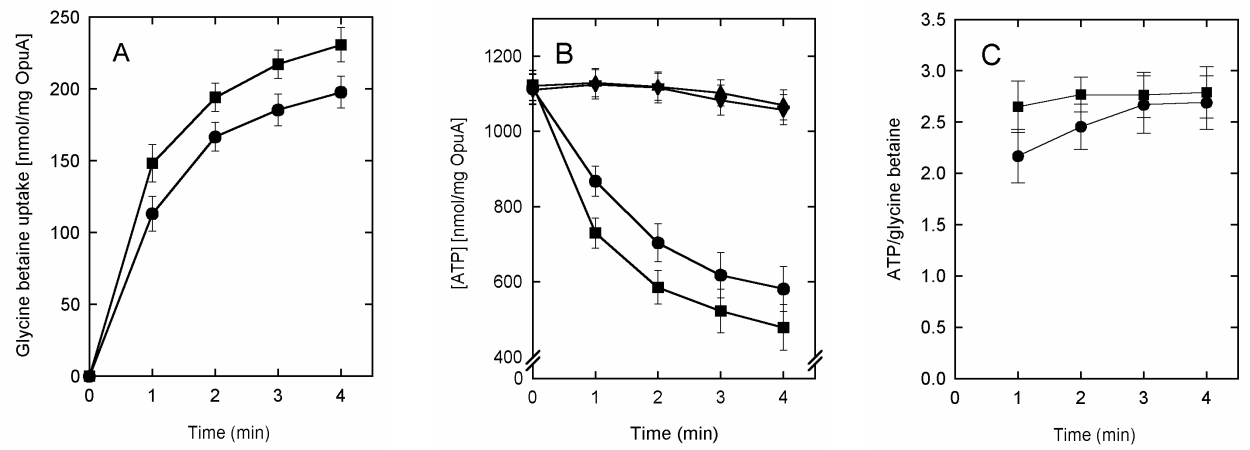

Fig. 2. Stoichiometry between ATP-hydrolysis and glycine betaine transport. A) Uptake of [^{3}H]-glycine betaine (final concentration, 63 µM,) was assayed in 50 mM KPi, pH 7.0, plus 200 mM (●) or 300 mM KCl (■). ATP and $MgSO_4$ were enclosed in the proteoliposomes. B) The ATPase activity was assayed in 50 mM KPi, pH 7.0, plus 200 mM (●,▲) or 300 mM KCl (■,▼) in the presence (●,■) or absence (▲,▼) of 63 µM glycine betaine. C) Calculated reaction-stoichiometries; the ATP/glycine betaine ratios were obtained from the data in panels A and B, and corrected for ATP-hydrolysis in the absence of glycine betaine.

Stoichiometry between ATP-hydrolysis and glycine betaine transport

To interpret the data concerning the catalytic cycle of ABC transporters, it is important to know how many molecules of ATP molecules are hydrolyzed per substrate molecule translocated, the so-called reaction-stoichiometry. For the analysis, it is desirable that the coupling between ATP-hydrolysis and substrate translocation is tight and that transport is unidirectional. The OpuA system meets these prerequisites. Fig. 2 shows a constant factor between the rate of glycine betaine uptake and the rate of ATP-consumption, reflecting a tight coupling between glycine betaine transport and ATP-hydrolysis throughout the course of the experiment. The ATP/substrate stoichiometry varied between 2 and 4 in separate experiments, implying that 2 to 4 ATP-molecules are hydrolyzed for each substrate-molecule translocated. The variation in the reaction-stoichiometry between separate experiments could be ascribed to differences in the ATPase activity, whereas the observed transport rates were highly reproducible. The ATPase activity of proteoliposomes was always negligible in the absence of glycine betaine.

Effect of GTP and ADP on the activity of OpuA

The advantage of the *in vitro* membrane-model system is that both translocation and ATPase activity can be followed in a manner that allows discrimination between inside-out and right-side out oriented transporters, enabling the distinction between osmotic effects at the "external" and "cytoplasmic" face of the transporter. Fig. 3 shows that right-side out oriented OpuA, energized by ATP inside the vesicle lumen, is activated when subjected to an osmotic upshift. With the ATP-regenerating system inside the proteoliposomal lumen, the rate of uptake levels off after a few min. After approximately 5 min, a plateau is reached and the uptake of glycine betaine is completely blocked even though residual ATP (±3 mM) is still present. Since efflux of glycine betaine is insignificant under these conditions, not only the overall influx but also the unidirectional influx must be completely inhibited. This inhibition is most likely caused by ADP, which is formed in the course of the experiment. Two pieces of information support this notion. First, the presence of 6 mM ADP strongly inhibits the transport of glycine betaine (Fig. 3; see below). Secondly, much higher values of glycine betaine are realized when the ATP-regenerating

system instead of ATP is enclosed in the proteoliposomes. Under these conditions, the ATP and ADP concentrations are kept high and low, respectively, for prolonged periods.

The inside-out oriented OpuA-molecules could be activated by the addition of ATP on the outside, thereby triggering efflux of glycine betaine from the vesicle-lumen. The inside-out oriented OpuA molecules could be activated by a high enough concentration of ionic osmolytes (KCl or NaCl) in the outside medium or in the presence of the amphipath tetracaine (see Chapter 4). In general, ABC transporters hydrolyze ATP to drive the transport reaction. It has been reported that other nucleotides, like GTP, can also be bound and hydrolyzed by the ATPase subunits. Indeed, Fig. 3 shows that GTP is capable of driving transport, albeit less efficient than ATP (Fig. 3). Furthermore, the addition of ADP in the outside medium inhibited the activity of OpuA, which is consistent with the notion that ADP is an inhibitor of ABC transporters.

Finally, from experiments as presented in Fig. 3, one can obtain an estimate of the fraction of right-side-out and inside-out-oriented OpuA molecules by comparison of the initial rate of uptake (via right-side-out OpuA) and efflux (via inside-out OpuA). Assuming that OpuA in both orientations is maximally activated, the analysis indicates that 76 ± 3 % is in the right-side-out-orientation.

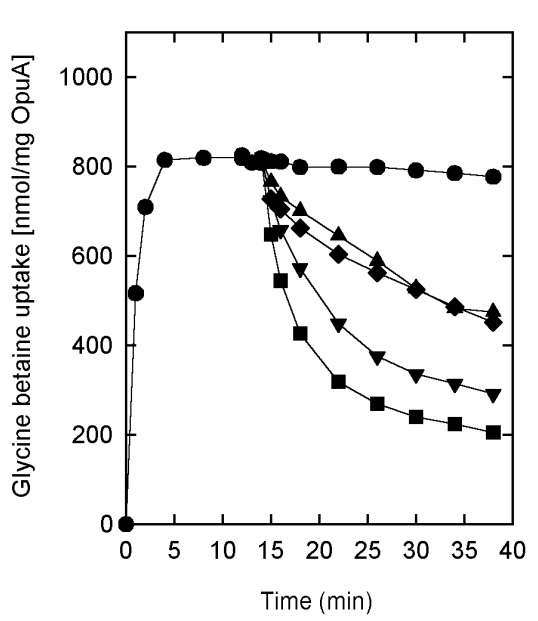

Fig. 3. The effect of several nucleotides on the activity of OpuA. Uptake of [^{14}C]-glycine betaine (final concentration, 63 µM,)(●) was assayed in 80 mM KPi, pH 7.0, plus 300 mM KCl. An ATP-regenerating system was enclosed in the proteoliposomes. After 14 min, Mg^{2+}/ATP (9 mM)(■), Mg^{2+}/GTP (9 mM)(◆), Mg^{2+}/ATP (3 mM)(▼) or Mg^{2+}/ ATP (3 mM) plus Mg^{2+}/ADP (6 mM)(▲).

Discussion

Two major unsolved questions in the field of ABC transport proteins relate to the directionality of transport and the reaction-stoichiometry, that is, the number of ATP molecules hydrolyzed per molecule of substrate transported. From a thermodynamic point of view, the hydrolysis of one ATP molecule per molecule of translocated substrate would be more than sufficient to explain the accumulation ratios observed *in vivo*. The $\Delta G_{ATP}/F$ in growing cells is in the order of -500 mV, which allows a more than 10^8-fold concentration of substrate with a reaction stoichiometry as low as 1. Assuming that ABC transporters generally operate in environments with (sub-)micromolar or higher concentrations of substrates, thermodynamic equilibrium would lead to molar concentrations of substrate in the cytoplasm. In practice, it is not desirable for transport systems to operate near equilibrium as this will slow down the net rate of transport. If some of the free energy difference is used to drive the reaction, the rate of transport will increase albeit at the expense of the efficiency of the process. The large free energy difference supplied by the hydrolysis of ATP, assuming a reaction stoichiometry of 1 or 2 ATP/substrate (see below), ensures a high rate of transport, even with submolar concentrations of glycine betaine in the cytoplasm. To address the questions regarding the reaction-stoichiometry, it is of major advantage that the purified and membrane-reconstituted ABC transporter facilitates a high rate of transport, and that substrate uptake is tightly coupled to ATP-hydrolysis. Membrane-reconstituted OpuA meets these criteria. Furthermore, the substrate-binding subunit of OpuA is fused to the

translocator subunit (53), thereby circumventing the 'problem' of having to add the substrate-binding protein separately.

For three ABC transporters, the general (AapJQMP) and branched-chain (BraDEFGC) amino acid permeases from *R. legiminosarum,* and the histidine permease (HisJQMP) from *S. typhimurium,* it has been reported that substrate transport is bi-directional. In whole cell experiments, evidence was obtained for substrate-binding protein-dependent uptake and efflux (58). The uptake and efflux of substrate are proposed to be independent processes, that is, efflux does not occur as part of an exchange cycle with incoming solute. The net substrate accumulation depended on the rate of uptake and efflux, and the final accumulation level was the result of equilibration of both processes. It has been reported that the maximal rates (V_{max}) of uptake and efflux are similar but that the affinity constants for uptake are 10^3-10^4-fold lower (58).

One of the proposed functions of the liganded substrate-binding protein is to transmit a signal via the transmembrane-spanning proteins to the ATPase subunits on the other side of the membrane. ATP hydrolysis then allows the opening of a translocation pathway concomitant with the release of the substrate from the substrate-binding protein (29). For the well-studied histidine and maltose ABC transporters from *S. typhimurium* and *Escherichia coli*, respectively, mutants have been isolated that displayed binding-protein independent transport with a low affinity, and a high intrinsic ATPase activity, indicating that ABC transporters supposedly have a low-affinity substrate-binding site within the transmembrane domain(s) (29;109). When accessible from the cytoplasmic side, such a low-affinity site could mediate the observed efflux of substrate. The substrate-binding protein dependence of efflux could relate to the signal transduction between the substrate-binding protein and the ATPase subunits.

Efflux of substrate was not observed when the HisJQMP complex was characterized in the membrane-reconstituted system, which can be explained by the fact that the final level of histidine accumulation (230 μM) was much lower than the presumed K_m for efflux (10.8 mM for the Aap system) (58;85). With membrane-reconstituted OpuA, we were able to accumulate glycine betaine to millimolar concentrations (± 30 mM). Under these conditions efflux of glycine betaine was still negligible. *In vivo*, glycine betaine can be accumulated to more than 0.5 M, which is two orders of magnitude higher than the highest intracellular levels effected by the glutamate ABC transporters from *R. legiminosarum* (58). Thus, unlike some other systems, OpuA seems to operate unidirectionally. At this moment, we cannot entirely rule out the possibility that additional cellular components modulate the activity of OpuA, and thereby cause efflux of substrate under *in vivo* conditions. We do not favor this possibility, and note that the *in vivo* experiments are more prone to artifacts, such as the presence of alternative systems that mediate the efflux of substrate.

In terms of understanding the catalytic mechanism of ABC transporters, it is of importance to know the reaction-stoichiometry. Our preliminary studies indicate that the ATP/glycine betaine stoichiometry is in the range of 2 to 4. The partially purified and membrane-reconstituted histidine transporter from *S. typhimurium* and maltose transporter from *E. coli* are reported to have a reaction-stoichiometry of 5 and between 1.4-17, respectively (7;28). It has been suggested for the histidine transporter that the spatial distance between the two lobes of the liganded binding-protein, which depends on the size/shape of the substrate, affects the interaction with the membrane complex, thereby influencing the reaction stoichiometry (87). Overall, there are no indisputable data on the coupling of ATP-hydrolysis to substrate translocation. The reported apparent reaction-stoichiometries cannot be easily understood in mechanistic terms. As many as two ATP molecules seem

most plausible because ABC transporters have two ATP-binding domains.

The proposed mechanism of substrate-binding protein-dependent ABC transporters can be summarized in a six-step catalytic-cycle model. In this model, it is proposed that two molecules of ATP are hydrolyzed in a single catalytic cycle, with one or two substrate molecules being translocated (for a full description of the model, see Chapter 1, section IV). How does a reaction-stoichiometry of 2 to 4 compare with this proposed mechanism? A value of 2 would be fully consistent with the model, while higher apparent stoichiometries may be explained by jamming of the system before the catalytic cycle is completed, that is, the transporter could be locked in a conformation where intrinsic ATPase activity can occur but translocation cannot be completed. The above presented model for the catalytic cycle describes the disengagement of the ATPase subunits upon binding of ATP, and an even further disengagement when the membrane complex interacts with liganded substrate-binding protein. In contrast to the *in vitro* situation, *in vivo* the eventual re-engagement of the ATPase subunits to the membrane complex may be facilitated by the high cytoplasmic protein concentration (macromolecular crowding), which affects the equilibria of oligomeric enzymes and thereby their function. Although it is unlikely that the function of the membrane-embedded proteins is affected directly by macromolecular crowding, the association with soluble or membrane-associated molecules such as the ATPase subunits of ABC transporters may be influenced (37;98).

Experimental Procedures

Bacterial strains, growth conditions, and isolation of membrane vesicles

Lactococcus lactis strain NZ9000 (30) was cultivated semi-anaerobically at 30 ºC in M17 broth, pH 6.5, supplemented with 1.0 % (w/v) glucose (GM17 medium) and 5 µg/ml chloramphenicol when carrying pNZopuAhis or derivatives. For the isolation of membranes, cells were grown in a 10 liter pH-regulated fermentor to an OD_{660} of 2, after which transcription from the *nisA* promoter was switched on by the addition of 0.2 % (v/v) culture supernatant of the nisin A producing strain NZ9700 (30). The final concentration of nisin A was about 2 ng/ml. The cells were harvested after one hour of induction, and inside-out membrane vesicles were prepared by lysing the bacteria (20 mg/ml) with a high pressure homogenizer (Kindler type NN2002; single passage at 10,000 p.s.i.), following (partial) digestion of the cell wall with 10 mg/ml lysozyme for 30 min at 30 °C (113). The membrane preparations were stored in liquid nitrogen.

Purification of OpuA

Membranes were resuspended in buffer A (50 mM KPi, pH 8.0, 200 mM KCl, 20 % glycerol) to a final concentration of 5 mg protein/ml and solubilized with 0.5 % *n*-dodecyl-ß-D-maltoside (DDM) for 30 min on ice. Following centrifugation, the solubilized material was incubated with Ni^{2+}-NTA resin (0.5 ml of resin/10 mg of membrane protein) for 2 hours at 4 ºC in the presence of 15 mM imidazole. Subsequently, the resin was washed with 20 column volumes of buffer A supplemented 0.05 % Triton X-100 and 15 mM imidazole. The his-tagged proteins were eluted from the column with 3 column volumes of buffer A supplemented with 0.05 % Triton X-100 and 200 mM imidazole.

Membrane-reconstitution of OpuA

Liposomes composed of the desired lipids were prepared, and membrane reconstitution was performed, essentially as described by Knol *et al.* (76). Briefly, preformed liposomes (4 mg/ml) were destabilized by titration with Triton X-100, and the turbidity of the suspension at 540 nm was used to monitor the physical state of the liposomes. Unless stated otherwise, liposomes destabilized to a point just beyond "detergent-saturation" (75) were mixed with purified OpuA in a 50:1 ratio (w/w), and incubated for 30 min at room temperature (RT) under gentle agitation. To remove the detergent, polysterene beads (Biobeads SM2) were added at a wet weight of 40 mg/ml, and the sample was incubated for another 15 min. Fresh Biobeads SM2 (40 mg/ml) were added to the sample four times and the incubations were continued at 4 °C for 15 min, 30 min, overnight, and 2 hours, respectively. Finally, the proteoliposomes were collected by centrifugation, washed twice with 50 mM KPi, pH 7.0, and stored in liquid nitrogen.

ATP-hydrolysis driven glycine betaine transport

An ATP-regenerating system was enclosed in the proteoliposomes by three cycles of freezing and thawing in the presence of KPi, pH 7.0 (50 mM), creatine kinase (2.4 mg/ml), ATP (6 mM), $MgSO_4$ (6 mM), and creatine-

phosphate (24 mM). Following extrusion of the proteoliposomes through a polycarbonate filter (200 nm pore size), the proteoliposomes were washed three times and resuspended in 80 mM KPi-buffer, pH 7.0 (iso-osmolar with the intra-liposomal medium; 180 mosmol/kg), to a concentration of 40 mg lipid/ml. The same procedure was followed when ATP (9 mM) plus $MgSO_4$ (9 mM) were enclosed on top of 50 mM KPi, pH 7.0. After washing, these proteoliposomes were resuspended in 50 mM KPi, pH 7.0 (iso-osmolar with the intra-liposomal medium; 115 mosmol/kg). Unless stated otherwise, the assay-buffer was pre-incubated at 30 ºC for 1 min in the presence of the desired concentration ([^{14}C]-)glycine betaine. To impose hyperosmotic conditions, additional salt or sugar was added to the medium. The transport reaction was initiated by the addition of proteoliposomes, which were diluted to a concentration of 1.8 mg lipid/ml.

(i) Transport assay. After given time intervals, 50 µl samples were diluted with 2 ml of ice-cold buffer of the same composition and osmolality as the assay medium, except that [^{14}C]-glycine betaine was omitted. The samples were filtered rapidly through 0.45 µm pore-size cellulose nitrate filters (Schleicher and Schuell GmbH, Dassel, Germany) and washed once more with 2 ml stop-buffer. The radioactivity on the filters was determined by liquid scintillation spectrometry. Duplicate samples were taken for analysis and each experiment was repeated at least twice.

(ii) ATPase assay. After given time intervals, 30 µl samples were drawn and assayed for ATP by using the ATPLite^{TM}-M system (Packard). Duplicate samples were taken for analysis and each experiment was repeated at least twice.

Materials.

M17 broth was obtained from Difco. Ni^{2+}-NTA resin was obtained from Qiagen Inc., Biobeads SM-2 from BioRad, *n*-dodecyl-β-D-maltoside from Sigma, Triton-X-100 from Boehringer Mannheim. Synthetic lipids were obtained from Avanti Polar Lipids. Radiolabeled [N-methyl-^{14}C] and [N-methyl-^{3}H] choline-chloride (55 mCi/mmol and 80 Ci/mmol, respectively) were obtained from Amersham (Buckinhamshire, England), and these precursors were used to synthesize [N-methyl-^{14}C]-glycine betaine and [N-methyl-^{3}H]-glycine betaine as described (81). Creatine kinase, creatine phosphate, and tetracaine were obtained from Sigma Chemical Co. All other chemicals were of reagent grade and obtained from commercial sources.

Miscellaneous

The osmolalities of media and buffers were measured by freezing point depression with an Osmostat 030 (Gonotec, Berlin, Germany). The protein concentration was determined by the method of Lowry *et al.* (89), using bovine serum albumin as a standard.

Acknowledgements

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ABC transporters: 1, 2 or 4 substrate-binding domains?

Tiemen van der Heide and Bert Poolman

Summary

Two families of ATP-binding Cassette (ABC) transporters have been detected, in which one or two substrate-binding domains are fused to either the N- or C-terminus of the translocator protein. Consequently, not one but two, or even four, substrate binding-sites are present in the functional ABC transporter complex.

The translocator of ABC transporters is composed of two transmembrane and two ATP binding domains (55). The individual domains can be expressed as separate polypeptides but they can also be fused to each other in any possible combination. In addition to these ubiquitous components, prokaryotic ABC transporters involved in solute uptake into the cell employ a specific ligand-binding protein or receptor to capture the substrate (Fig.1). These substrate-binding proteins were first identified in Gram-negative bacteria, where they reside in the periplasmic space (147). Later, it was discovered that Gram-positive bacteria, organisms without a periplasm, anchor the proteins to the outer surface of the cell membrane via an N-terminal lipid moiety (42;145). This glyceride lipid modification at the amino-terminal cysteine has also been observed for exofacial substrate-binding proteins in Archaea (Fig.1A). However, these microorganisms also use the strategy of an N-terminal transmembrane segment to anchor the protein to the cytoplasmic membrane (2),

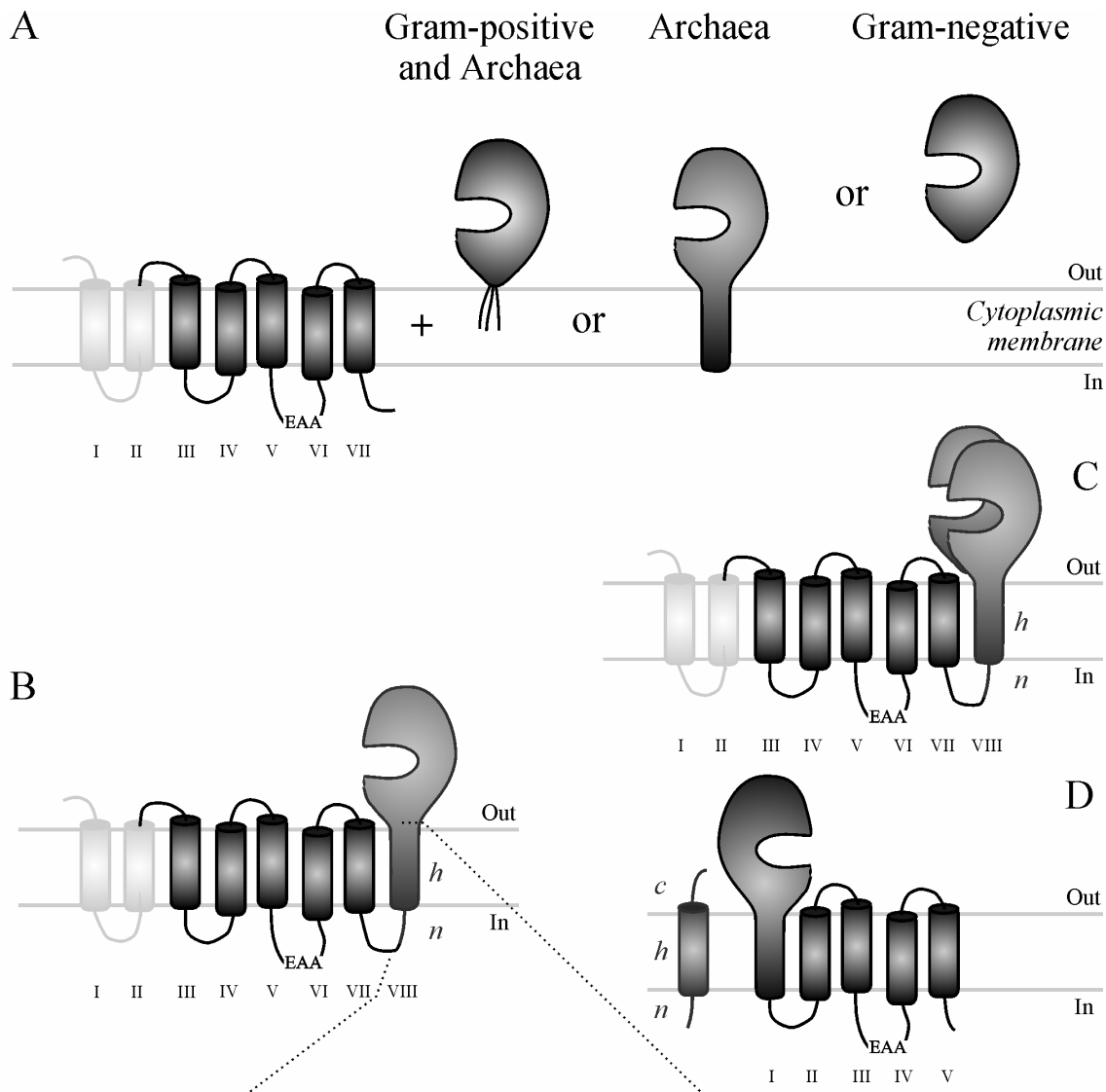
Chimeric receptor/translocator proteins

The ABC transporter for glycine betaine from *Lactococcus lactis* has the substrate-binding moiety fused to the C-terminus of the translocator domain (53;104) and both termini are located at the outer surface of the membrane (for detailed information, see Chapter 1). Together with the ATPase subunit (OpuAA) the chimeric receptor/translocator protein (OpuABC) constitutes the OpuA complex, which belongs to the OTCN family of the ABC superfamily (26). By performing Blast searches of the non-redundant database (NCBI) with the OpuABC protein from *L. lactis* and manual inspection of ABC operons in published genome sequences (http://www.ncbi.nlm.nih.gov/PMGifs/Genomes.micr.html), we show that similar systems are present in several families of low-GC Gram-positive bacteria (*Clostridiaceae, Listeriacea, Streptococcaceae*, *Staphylococcaceae)*, high-GC Gram-positive *Actinobacteria* (*Streptomyces*), and the Gram-negative *Helicobacteraceae* within the *ε-Proteobacteria* (Table I). OpuA from *L. lactis* is the only well-studied representative of this subset of ABC transporters with this novel architecture (53;54;104). Database searches also indicate that within the PAO family of the ABC superfamily, comprising putative glutamine/glutamate transporters, the substrate-binding protein is fused to the N-terminus of the translocator. This architecture of ABC transporters was discovered in the heretofore-named families but also in *Cyanobacteria* (*Nostoc* and *Synechocystic sp.*).

By analogy with other ABC transporters, the chimeric systems will be composed of two integral membrane subunits and two ATP-binding subunits. This oligomeric structure implies that two receptors are present per functional complex. Even more surprising than a single receptor fused to a translocator unit is the finding that *opuABC* from *S. coelicolor A3* codes for a protein with two copies of the substrate-binding domain fused to the C-terminus of the translocator (Fig.1C). Like OpuABC from *L. lactis*, this protein belongs to the OTCN family and is a putative glycine betaine transport-component. Similarly, in *L. lactis, S. pneumonia*, *S. pyogenes* and *S. agalactiae,* members of the PAO family have two copies of the substrate-binding moiety fused to the N-terminus of the corresponding translocator domain. If these systems form a dimeric complex, four binding domains are present per functional unit. Alternatively, these proteins could form a heterodimeric complex with a transmembrane subunit lacking a substrate-binding domain. Genome searches indicate that each of the genes specifying a chimeric two-receptor/translocator protein is present in an operon with the gene(s) for the ATPase subunit(s), but additional transmembrane components are absent, favoring an architecture with four binding domains. In a few cases, a homologue of the corresponding transmembrane domain of the chimeric protein can be found elsewhere on the chromosome.

It is possible that the two substrate-binding domains within one polypeptide have evolved from gene duplication. Analysis of the primary sequences of the two-receptor/translocator proteins, belonging to the PAO family, from *S. pneumonia*, *S. pyogenes* and *S. agalactiae* showed that the substrate-binding domains within one polypeptide are on average 45 % identical. A similar identity-score was found when the substrate-binding domains of the three polypeptides from *S. pneumonia*, *S. pyogenes* and *S. agalactiae* were compared, while the translocator domains of these proteins are on average 84 % identical. Similar identity-scores were found when the polypeptides containing one substrate-binding domain were compared with each other. However, when polypeptides with one and two binding-domains in one and the same organism were compared, as could be done for *S. pneumonia* and *S. pyogenes*, the substrate-binding and translocator domains shared on average 25 % and 35 % identity, respectively. Two models could explain the low identity between the polypeptides with one and with two substrate-binding domains. Firstly, the polypeptides do not have the same ancestor and are involved in different transport processes. Secondly, polypeptides with one and two substrate-binding domains have the same ancestor, and transport similar substrates, but the proteins have evolved/adapted differently in order to function with two or four substrate-binding sites, respectively.

The SignalP prediction method (http://www.cbs.dtu.dk/services/SignalP-2.0/)(102) indicates that the N- and C-terminal receptor domains are preceded by typical bacterial signal sequences (Fig.1E). The N-terminal signal sequence is most likely processed by a signal peptidase, resulting in a substrate-binding domain with a free N-terminus. In contrast, the segment preceding the C-terminal receptor domain(s) has an anchoring function and is not cleaved (5). The presence of this signal anchor sequence suggests that these chimers have evolved from the fusion of a gene for a pre-protein to the 3' end of a translocator gene. The sequence identity among the signal anchor sequences (Fig.1E) is low compared to that of other TMS within the same family, suggesting that it is not an inherent part of the translocation pathway.

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ChoS_Ll     : SLLKFLEKAK IKTILISFFIGLLVLGGSY  YRPQTSNHPQ: 234
OpuABC_Ll   : NPTVKKWKRG IALVSLLALIIGAFSGMS   FGKTASDKKV: 323
ProWX_Spn   : LKVMEKAKLR TIFSGFALVALLLGLSYSP  ALLVQKEKEN: 236
OpuABC_Spy  : KAKAGKTNKW IGLAALAVFLIAALGRGIM  AMTSGMADKG: 321
OpuBB_Lm    : RFLEKASFKS TIITISAGILLTAAIIVVPY FASDKKEITV: 238
lin1461_Li  : RFLEKASFKS TIITISAGILLTAAIIVVPY FASDKKEITI: 238
SA0678_Sau  : RFMAKLSYKK LLMTLGVIVMIIILAIAIPM FAQKGDKITL: 238
CAC2849_Ca  : LKKGRNKKYK IVAAILIVITIISGSIMFY  NSQNKKVIVI: 261
CPE0558_Cp  : NKKKNTFVLK IISVIMIIAILFMGISSFI  SSRKDKVVIG: 252
ProWX_Hp    : NATKKQKRRV YTNLAVFLFLLLASALWLI  PRNAIEEKPL: 289
OpuABC_Sco  : KGLKIWSYRP QPQVAVVGIVILALVAGGM  GMFGGSDDAD: 326
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Fig. 1. Schematic representation of the architecture of the substrate-binding and translocator moieties of ABC transporters. The cylinders depict the transmembrane segments (TMS). The chimeric proteins with receptor at the C-terminus (B, C) have an additional TMS, denoted here as VIII, and the corresponding substrate-binding moieties together with the signal anchor are indicated in blue. The signal sequence of the chimera with the receptor at the N-terminus is most likely cleaved off (D). Since the first two TMS are not always present, they are indicated in grey (A, B, C). The position of the signature motif (EAA) of the ABC translocator proteins is also depicted. Panel E shows the alignment of the C-terminal signal anchors; the underlined residues are predicted cleavage sites but for OpuABC it has been demonstrated that cleavage does not occur (54).

Table 1. OTCN and PAO family members. The number under trans-membrane segments includes the signal anchor segment in case of the OTCN members. Predicted signal peptide cleavage site and mean signal peptide score were obtained from the SignalP prediction method (102). A signal (anchor) sequence is predicted for each of the proteins.

Organism	Gene	Family	Protein size (amino acids)	Accession number	*Trans*-membrane helices	Substrate binding domains	Predicted signal-peptide cleavage-site	Mean signal peptide score (mean S)
Lactococcus lactis	*opuABC(busAB)*	OTCN	573	NP_267607	8	1 on C-terminus	yes	0.859
	choS	OTCN	500	NP_267004	6	1 on C-terminus	no	-
	glnP	PAO	714	NP_267915	5	2 on N-terminus	no	-
Streptococcus agalactiae	*glnP*	PAO	727	AAK57382	5	2 on N-terminus	no	-
Streptococcus pneumonia	*proWX*	OTCN	506	NP_359269	6	1 on C-terminus	yes	0.866
	sp0543	PAO	521	NP_344974	5	1 on N-terminus	no	-
	sp1241	PAO	721	NP_345706	5	2 on N-terminus	no	-
Streptococcus pyogenes	*opuABC*	OTCN	575	NP_268563	8	1 on C-terminus	yes	0.749
	spy0277	PAO	522	NP_268629	5	1 on N-terminus	no	-
	spy1315	PAO	724	NP_269437	5	2 on N-terminus	no	-
Staphylococcus aureus	*sa0678*	OTCN	504	NP_373933	6	1 on C-terminus	yes	0.903
	sa1675	PAO	441	NP_374965	5	1 on N-terminus	no	-
Listeria innocua	*lin1461*	OTCN	504	NP_470797	6	1 on C-terminus	yes	0.785
	lin0840 and *lin2352*	PAO	480 and 486	NP_470182 NP_471683	5	1 on N-terminus	no	-
Listeria monocytogenes	*lmo1422*	OTCN	504	NP_464947	6	1 on C-terminus	yes	0.786
	lmo0847 and *arpJ*	PAO	480 and 486	NP_464373 NP_465774	5	1 on N-terminus	no	-
Clostridium acetobutylicum	*cac2849*	OTCN	523	NP_349452	6	1 on C-terminus	no	-
	cac0111	PAO	477	NP_346756	5	1 on N-terminus	no	-
Clostridium perfringens	*cpe0558*	OTCN	517	NP_561474	6	1 on C-terminus	yes	0.903
Streptomyces coelicolor	*opuABC*	OTCN	871	CAB59473	8	2 on C-terminus	no	-
Helicobacter pylori	*hp0818*	OTCN	553	NP_207611	6	1 on C-terminus	yes	0.897
Nostoc sp.	*alr3187*	PAO	501	NP_487227	5	1 on N-terminus	no	-
Synechocystic sp.	*glnH/glnP*	PAO	530	NP_440904	5	1 on N-terminus	yes	0.902

Biological significance

The functional role of two or perhaps four substrate-binding domains per functional complex represents an intriguing problem, as it is generally assumed that a single substrate-binding protein interacts with the translocator. The discovery of chimeric substrate-binding/translocator proteins provides new insights into the functioning of these ABC transporters. It seems likely that multiple binding domains would increase the efficiency of the translocation process or broaden the specificity of the system, that is, the substrate-binding domains fused in tandem could have a different specificity. Two models could explain the benefit of having two (or more) substrate-binding domains in terms of translocation efficiencies. First, the 2nd binding domain could deliver the substrate to the one recruited by the membrane complex, implying that the domains have a different function in the translocation process. This would be equivalent to the situation in Gram-negative bacteria where most, if not all, substrate-binding protein-dependent ABC transporters seem to have the periplasmic substrate-binding protein in excess of the translocator components (4). Secondly, the binding domains could alternately interact with the membrane complex. In this view, the 2nd (and/or 3rd and 4th) domain could already capture a substrate molecule for a subsequent cycle of translocation, while the first binding-domain is still docked onto the membrane-embedded translocator complex. Under conditions that substrate binding is rate-determining, the rate of transport would be increased by the presence of additional substrate-binding sites.

It is interesting to note that the chimeric receptor/translocator proteins have been found in only two families of the ABC superfamily, and the systems are predicted to transport glycine betaine (or related compounds) or glutamate/glutamine. The accumulation levels of these solutes are among the highest found in bacteria, *e.g.* glycine betaine can be present at molar level (111), which requires high rates of transport. The high levels of accumulation of these solutes relate to their role as osmoprotectants.

Finally, the glutamate receptor channels, which are part of the predominant excitory neurotransmitter system in the vertebrate brain, have ligand-binding moieties distantly related to the glutamate-binding proteins of ABC transporters (105). Although these channels are unrelated to the ABC transporters, they may have the receptor moiety linked and communicating with the translocator in a manner similar to the systems described here.

Acknowledgements

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Discussion and concluding remarks

General introduction

In their natural environment, microorganisms can be subjected to osmotic stress, which is the result of a change in the water activity of the external medium. Since the cytoplasm of microorganisms is separated from the external medium by a semi-permeable lipid membrane, only permeable to water and small hydrophobic molecules, water influx or efflux occurs when the medium osmolality is decreased or increased, respectively. When subjected to an osmotic upshift, the cell is dehydrated and the turgor decreased. Turgor is the outward pressure acting on the cell wall as a result of the osmotic imbalance between intra- and extracellular fluids. Since turgor supplies the force needed for expansion of the cell, it is of major importance to maintain turgor above a critical value. This is achieved via the accumulation of compatible solutes, which can be accumulated to high intracellular concentrations without affecting vital cellular processes. To enable and maintain the necessary high gradients of compatible solutes across the cytoplasmic membrane, the bacteria deploy transport systems that use ATP-hydrolysis or electrochemical ion gradients across the membrane to drive the uptake reaction. Upon a decrease of the medium osmolality, referred to as osmotic downshift, the cell has to deal with a movement of water into the cell that results in an elevation of the turgor. When subjected to a severe osmotic downshift, the subsequent increase of turgor can lead to cell lysis by rupturing of the cell wall. Cell lysis is prevented by the rapid expulsion of solutes via the opening of mechanosensitive channels in the membrane.

At the onset of the work described in this thesis, it was clear that microorganisms are responding to an osmotic upshift by increasing the capacity to accumulate compatible solutes by increasing the expression of genes coding for osmoregulated transporters and increasing the activity of systems already present in the membrane. However, the osmotic signal(s) sensed by bacteria when subjected to an osmotic upshift was unknown, as was the mechanism by which this signal was transduced to the osmotically activated transporters.

A detailed study of the osmoregulated ATP-binding cassette (ABC) transporter OpuA from the Gram-positive lactic acid bacterium *Lactococcus lactis* was initiated to gain further insight into the osmosensing mechanism(s).

Osmotic signaling and omosensing mechanism(s) of OpuA

To gain more insight into the mechanism rooting the osmotic activation of OpuA and to prove that this ‘two-component ABC transporter’ is functional as osmosensor and osmoregulator, the proteins were overexpressed, purified and incorporated into ‘artificial membranes’, so-called proteoliposomes (Chapter 3). Initial experiments showed that OpuA could be osmotically activated by a *trans*-membrane osmotic gradient (outside osmolality high relative to the inside). The activation by an osmotic upshift was instantaneous (< 1 sec), fully reversible, and followed the changes in volume of the proteoliposomes (Chapter 4). This demonstrated that the two components OpuABC (substrate-binding domain + translocator domain) and OpuAA (ATPase), constituting the OpuA system, are sufficient for osmosensing and osmoregulation.

The fact that osmotic activation of OpuA in proteoliposomes mimics the regulation *in vivo* might seem surprising, as the vesicles do not

withstand large pressures whereas the turgor in cells is several atmospheres. However, there is evidence suggesting that in bacteria turgor exists across the cell wall (and outer membrane in case of Gram-negative bacteria) and not across the cytoplasmic membrane (17). This implies that osmosensing devices present in the cytoplasmic membrane cannot respond to changes in turgor pressure, rather they must sense the consequences of the water influx or efflux, *e.g.* changes in ionic strength. Proteoliposomes behave osmo-metrically, that is, water diffuses across the membrane in response to an osmotic imbalance, which leads to a decrease in the volume to surface ratio when the outside osmolality is increased. The accompanying changes in the membrane structure and the concentration of the contents of the lumen may be compared with those in cells that are in a state of plasmolysis (156). The consequences of osmotic shifts, experienced by proteins in membrane model systems such as proteoliposomses, may thus be similar to those in the *in vivo* situation.

It was observed that proteoliposomes with an average diameter of 200 nm change their shape from spherical to sickle-shaped when subjected to an osmotic upshift (Fig. 1). This macroscopic deformation of the membrane is not critical for the osmosensing and regulatory mechanism of OpuA (Chapter 4). Hence, in contrast to the osmotic downshift-activated mechanosensitive channel proteins, there is currently no evidence that OpuA or other osmotic upshift-activated systems are sensitive to "physiologically relevant" mechanical deformations of the membrane. The decrease of the volume to surface ratio of the proteoliposomes upon osmotic upshift results in an elevation of the internal osmolality and ionic strength. In Chapter 4 and 5, it is described that the increase in the ionic osmolyte concentration (or ionic strength) at the cytoplasmic face of OpuA is responsible for the activation, demonstrating that a change in the intracellular ionic strength, rather than a specific signaling molecule or change in water activity, is the primary osmotic signal.

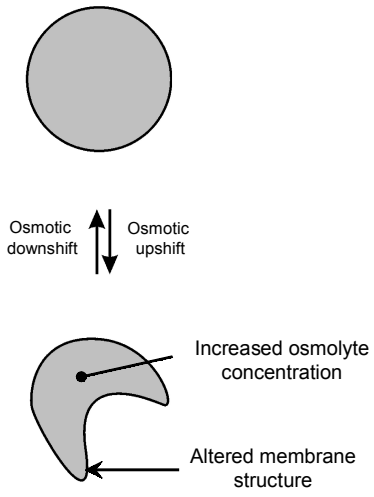


Fig.1. Proteoliposomes change their shape from spherical to sickle-shaped when subjected to an osmotic upshift. This changes the membrane structure and internal osmolyte concentration.

How then does OpuA sense this osmotic signal? As the transporter is membrane-embedded, it has intimate contact with lipids in order to form a tight seal with the membrane. Thus, the lipid bilayer seems a logical candidate that could act as a mediator to communicate the osmotic signal via changes in the lipid/protein interactions. Consistent with this idea is the observation that low concentrations of small lipophilic amphipathic molecules, which interact with the membrane, mimic the osmotic-upshift activation of OpuA (Chapter 3). Similar observations have been reported for the osmotic-upshift activated electrochemical ion gradient-driven transporter BetP from *C. glutamicum* (129).

To investigate the role of the membrane as a possible transducer of the osmotic signal, OpuA was incorporated into liposomes of different lipid composition (Chapter 4). For the interpretation of the data, the assumption was made that differences in the minimal osmolality needed for activation of OpuA would be indicative of altered osmosensing, whereas variations in the maximal uptake rates are not. The validity of this approach is partly rooted in the findings that the maximal rates of non-osmoregulated transporters from *L. lactis* (and related organisms) are similarly dependent of lipid composition as OpuA, *e.g.* anionic and non-bilayer lipids are critical for activity and

optimal activity is reached with C_{16} or C_{18} acyl chains (unpublished results on the lactose transport system of *Streptococcus thermophilus*)(60). The fraction of anionic (charged) lipids was found to be of major importance for the osmotic activation of OpuA, whereas variations in acyl chain length, position and configuration (*cis/trans*) of the double bond, and fraction of non-bilayer lipids had relatively minor effects, making it unlikely that osmotic stress is detected as a perturbation of the lateral pressure profile. Although supported by a less elaborate set of experiments, a similar conclusion can be reached for the BetP glycine betaine transporter from *C. glutamicum* (129). The measurements in the proteoliposomes with varying lipid (and amphipath) composition strongly suggest that the overall charge of the headgroup region of the membrane lipids determines the activity (kinetic state) of the OpuA transporter.

Overall, the presented data suggest that OpuA is under the osmotic control of a mechanism in which the membrane and ionic strength act in concert to signal osmotic changes (Fig. 2). Future measurements of the membrane surface charge might yield valuable information on how the electrostatics of the membrane varies as a function of the ionic strength and/or lipid composition.

Osmotic regulation of other transport systems and osmosensing kinases

Also for other osmoregulated transport systems, attempts have been made to identify the primary osmotic signal that triggers the activation. For the transporter BetP from *C. glutamicum,* it has been concluded that the protein is a sensor of cytoplasmic potassium because K^+ and the analogs Rb^+ and Cs^+ activate the system, whereas organic cations and NH_4^+ do not (128). However, since BetP is driven by an electrochemical gradient of sodium, the authors have not been able to test Na^+ (and the analog Li^+). This leaves open the possibility that BetP is actually sensing inorganic cations rather than specifically K^+. To

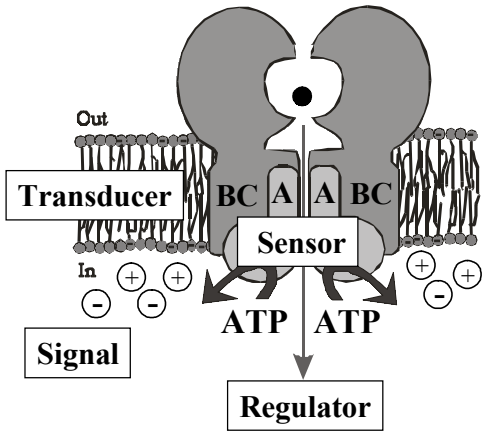


Fig. 2. Osmosensing and osmoregulatory mechanism of OpuA.

verify the postulated K^+-sensing mechanism of BetP, further experimentation is needed, *e.g.* the isolation of mutants and kinetic experiments to discriminate between K^+ and Na^+.

For the proline transporter ProP from *E. coli* (or *Salmonella typhimurium)*, the osmotic activation has not specifically been studied as a function of osmolyte concentration and composition at the cytoplasmic face of the protein. However, the reported *in vitro* data (96;117) are consistent with a mechanism in which the ionic osmolyte concentration or ionic strength serves as an osmotic signal. *In vivo*, ProP from *S. typhimurium* is activated by an osmotic upshift but the system stays active even after potassium uptake has ceased (78). The decrease in potassium uptake is thought to reflect restoration of turgor, but the cells must still be in a state of stress, as ProP is not deactivated under these conditions. Since the initial response of *E. coli* and *S. typhimurium* involves the accumulation of potassium ions, it is well possible that the increased electrolyte concentration keeps the ProP system in the activated state.

Similar to BetP from *C. glutamicum* (128), ProP catalyzes bi-directional transport, which complicates the measurements of osmotic activation, in particular when the protein is randomly inserted in the membrane and/or the orientation after reconstitution is unknown. The ABC transporter OpuA, on the other hand, catalyzes unidirectional transport and the fraction of inside-out oriented molecules does

not contribute to the observed activity when ATP is only present in the lumen of the vesicles. In the case of OpuA, ATP-driven uptake of glycine betaine by right-side-in and ATP-driven glycine betaine efflux by inside-out reconstituted protein has been measured, which allowed rigorous discrimination between effects exerted at the cytoplasmic and external face of the transporter (Chapter 4 and 5). Moreover, it was possible to determine the ATPase activity of the membrane reconstituted system.

For two sensor kinases, the histidine kinase KdpD and the sensor kinase EnvZ from *E. coli*, also an elaborate set of data on osmosensing is available. KdpD is needed for the osmo-regulated expression of the *kdpFABC* operon, which encodes a P-type potassium-ATPase, at limiting concentrations of potassium. The osmotic activation of this kinase also involves sensing of the cytoplasmic ionic strength, but this system has an additional, specific, mechanism to sense K^+ (68;140). The sensor kinase EnvZ is involved in the osmoregulated expression of both OmpC and OmpF (outer membrane porins). EnvZ, reconstituted in proteoliposomes, is activated by K^+, Na^+, Rb^+ and NH_4^+, irrespective of the counterion (Cl^-, Br^-, SO_4^{2-}, NO_3^- or glutamate), whereas neutral osmolytes have no effect (67). Although the highest stimulation of autokinase activity in this membrane-reconstituted system was achieved with K^+ as the cation, the data are in agreement with the proposal for the OpuA ABC transporter and the KdpD sensor kinase, that is, the cytoplasmic face of EnvZ senses the concentrations of ionic osmolytes.

Taken together, the data from *in vivo* and *in vitro* studies indicate that external ionic and nonionic osmolytes activate a range of osmosensing devices, provided the compounds do not equilibrate (cross the membrane) on the time scale of the measurements. This osmotic stress causes the cytoplasmic or liposomal volume to decrease, resulting in an increased internal osmolyte concentration. *In vivo*, not only the decrease in cell volume but also the accumulation of potassium ions in the initial response to osmotic upshift may lead to large increases in the cytoplasmic ionic osmolyte concentrations. Since the *in vitro* studies with OpuA clearly discriminate between ionic and nonionic osmolytes at the cytoplasmic face of the protein, ionic osmolytes (or ionic strength) are postulated to serve a role as the primary signal under hyperosmotic stress. This signal may not be sensed directly by the protein*, i.e.,* via changes in surface hydration of the proteins or specific binding of ions by the protein. Rather, the membrane in which the protein is embedded is postulated to serve as mediator. Changes in cellular ionic strength are likely to alter specific interactions between (ionic) lipids and the proteins, thereby altering their activity.

Rationale for ionic strength as the osmotic signal

Why would the cell use ionic osmolytes rather than intracellular osmolality (affecting protein hydration) or a specific signaling molecule (allosteric regulatory site on the protein)? When the medium osmolality is raised, the initial change in cytoplasmic water activity depends on the elasticity of the cell wall. Contrary to what is often thought, the cell wall of bacteria is not rigid but actually quite elastic (22;34). Consequently, even at turgor values above zero, the cytoplasmic volume decreases with increasing external osmolality, and the ion (osmolyte) concentrations increase accordingly. The increase in ionic strength accompanying the volume decrease is undesirable as too high concentrations of electrolytes interfere with macromolecular functioning in eubacteria as well as higher organisms (160). As well documented for *E. coli* (33;56;122), most eubacteria expel ionic compounds in the event the electrolyte concentration becomes too high and replace these molecules with neutral osmolytes such as glycine betaine to balance the cellular osmolality. The increase in electrolyte concentration (or ionic strength) upon a modest decrease in turgor pressure would thus represent

an excellent trigger ("osmotic signal") for the activation of any osmoregulated transporter of neutral compatible solutes, such as OpuA. Actually, it would prevent the osmotic stress from turning into "electrolyte stress".

Why, then, is the increase in intracellular osmolality less suitable as osmotic signal? In order to maintain a relatively constant turgor at different external osmolalties, the cell must be able to switch on transporters and take up organic compatible osmolytes with maximal activity at different internal osmolalities. In other words, the ability of (the majority of) microorganisms to grow at their maximal rate over a wide range of medium osmolalities implies that cellular processes function optimally over a wide range of intracellular osmolalities. Finally, specific signals may be used to tune the activity or expression of a particular system, as suggested by the data on the sensor kinase KdpD (66). However, ionic strength seems a more general signal for osmoresponsive systems, including signal transduction pathways to control the expression of osmoregulated genes.

Directionality and reaction-stoichiometry of ABC transporters

In vivo studies on substrate-binding protein-dependent ABC-transport suggests that some systems display both uptake and efflux of substrate (for detailed information, see Chapter 1 and 5). If one believes in the conservation of structure and function in biology, the observed bidirectional solute movement might be a general feature of the transport members of the ABC-superfamily. In Chapter 5, it is reported that the osmoregulated ABC transporter OpuA operates unidirectional in the *in vitro* membrane-model system. At this moment, we cannot entirely rule out the possibility that additional cellular components modulate the activity of OpuA and other ABC transporters, and thereby cause efflux of substrate *in vivo*. However, we do not favor this idea and believe that the *in vivo* experiments are more prone to artifacts, such as the presence of alternative systems that mediate the efflux of substrate, than the *in vitro* measurements.

The unidirectional transport of glycine betaine by OpuA was an important advantage in the determination of the reaction-stoichiometry of the system, that is, the number of ATP-molecules hydrolyzed per substrate molecule translocated. In Chapter 5, reaction-stoichiometries for OpuA of 2 to 4 are reported. A stoichiometry of 2 would be in agreement with the proposed model for the catalytic mechanism of substrate-binding protein-dependent ABC-transport, while higher values cannot be easily understood in mechanistic terms. The variation in the reaction-stoichiometry between separate experiments could be ascribed to variations in the ATPase activity, whereas the observed transport rates were highly reproducible (for further details, see Chapter 1 and 5).

Structural architecture of substrate-binding protein-dependent ABC transporters

In the classical view, substrate-binding protein-dependent ABC transporters are typically composed of five protein(s) (domains), that is, an extracellular substrate-binding protein, two ATP-binding/hydrolyzing subunits and two integral membrane subunits. Genomic database searches revealed that in several groups of bacteria a subset of ABC transporters is present that have one or even two binding-proteins fused to either the C- or the N-terminus of the integral membrane subunit, resulting in one polypeptide that comprises the substrate-binding domain(s) and the translocator moiety (Chapter 6). OpuA is the only well-studied representative of this subset of substrate-binding protein-dependent ABC transporters. By analogy with other systems, this subset of ABC transporters is most likely functional as a dimer, which would result in a functional complex with multiple substrate-binding domains present. Models that might explain the advantage of having multiple substrate-binding domains in

the functional ABC transporter complex are discussed in Chapter 6 of this thesis.

Concluding remarks and future perspectives

At present, it is unclear whether the ATP-binding/hydrolyzing subunit (OpuAA) or the membrane-embedded part (translocator; OpuABC) of OpuA is the actual osmo-sensor. A challenge will now be to assign specific regions or residues in the OpuA complex as sites that actually sense the changes in membrane structure (*e.g.* electrostatic interactions between protein and phospholipid molecules), and to translate the *in vitro* observations into proposals that can be experimentally tested *in vivo*. As the activation of OpuA by a rise of the concentration of ionic osmolytes at the cytoplasmic face of the complex is modulated, it seems most likely that the sensory part of OpuA resides in the inner membrane leaflet.

To gain a full understanding of the response of bacteria to an osmotic upshift, one may wish to study the osmotic signaling pathways that affect the transcription of genes. It will also be of interest to assess further the influence of the membrane on the osmotic activation of OpuA. As the membrane bilayer composition is intrinsic to the osmosensing mechanism of OpuA and most likely other transporters as well (117;129), changes therein will on the longer time scales be important for volume control of the cell. Therefore, one would also like to know how the cell responds to osmotic stress, not only at the level of activation of transcription and transporter activity, but also at the level of lipid synthesis. Preliminary studies have been reported on the changes in fatty acid composition of the membrane of *L. lactis* in relation to osmotic stress (47). Unfortunately, no information is available on variations in head group compositions, *c.q*. fractions of anionic lipids, as these factors seem far more important for the osmoregulation of OpuA than acyl chain length, degree of saturation etc. Finally, the relationship between osmotic and other stresses needs to be evaluated more thoroughly at the level of transporter activity (*in vitro* and *in vivo*) and cellular glycine betaine accumulation levels.

Over the last few years, medium and low-resolution structures of membrane proteins have been presented. Of major interest for the field of ABC transporters are the low-resolution structures of the human multi-drug resistance (MDR) ABC transporters P-glycoprotein and MRP1 (126;127), the human antigen presenting TAP transporter (153), and the bacterial MDR ABC transporter MsbA (18), which suggest allosteric movements of domains during catalysis. Furthermore, some high-resolution structures allowed the visualization of protein-associated lipid molecules (94;149). These structures were determined with X-ray diffraction methods that only detect highly ordered lipid structures. Since lipids are known to exchange rapidly at the protein-lipid interface (63)(135), it remains to be established if the crystallographic analyses contribute to our understanding on how lipids affect the functioning of membrane proteins such as OpuA. For a good interpretation of the structural information, more kinetic data on membrane-reconstituted proteins in relation to the membrane lipid composition are needed. In this regard, the developments in the field of molecular dynamics that deal with lipid/lipid and lipid/protein interactions are also important.

Nederlandse samenvatting voor niet biologen

1. Wat is het belang van het bestuderen van bacteriën?

'Wat is het nut van het bestuderen van bacteriën?' Met deze vraag zal waarschijnlijk elke (moleculair) microbioloog wel een keer geconfronteerd zijn. Een bacterie is niet met het blote oog zichtbaar en het is voor de meeste mensen dan ook moeilijk voor te stellen dat bacteriën een essentiële rol spelen in het handhaven van het ecosysteem. Ze zorgen voor de recycling van belangrijke bouwstoffen en spelen een grote rol in het verteringsstelsel van mens en dier. Bacteriën zijn tevens de evolutionaire voorlopers van al het leven op aarde. Hun bestudering levert dan ook inzicht in de universele principes die ten grondslag liggen aan vele processen die zich afspelen in de cellen van micro-organismen, planten, dieren en mensen.

Een bacterie is het kleinste zelfstandige organisme die alle functies bezit om te overleven in een onvoorspelbare omgeving, en onder de juiste condities groeit en vermenigvuldigt een bacterie zich snel. Dit gebeurt a-sexueel, wat inhoudt dat de 'nakomelingen' exacte kopieën zijn (klonen) van de moedercel en dat de genetische opmaak van alle cellen identiek is. Tevens is het een groot voordeel dat de genetische code van bacteriën eenvoudig te modificeren is. Dit schept de mogelijkheid om gericht een eigenschap toe te voegen of uit te schakelen. De voorgenoemde eigenschappen maken dat bacteriën een favoriet modelorganisme zijn in laboratoria.

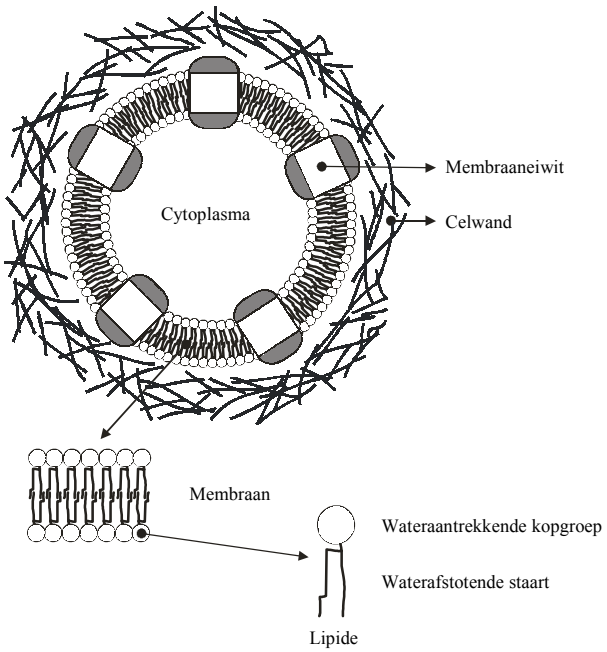
De flexibiliteit van bacteriën die ervoor zorgt dat ze kunnen overleven onder bijna alle condities heeft echter ook tot gevolg dat ze de oorzaak kunnen zijn van voedselbederf en bacteriële infecties. Binnen de voedingsindustrie zijn een aantal strategieën ontwikkeld om voedselbederf te voorkomen, zoals het steriliseren en het met UV-licht bestralen van voedsel. De consument staat echter wantrouwig tegenover UV-bestraald voedsel en sterilisatie gaat ten koste van de smaak. Ook in de gezondheidszorg kunnen bacteriën voor grote problemen zorgen. Het recente verleden leert dat een steeds groter aantal bacteriën een weerstand tegen allerlei antibiotica ontwikkelt. Hierdoor zijn bacteriële infecties steeds moeilijker te bestrijden (een bekend voorbeeld is de "ziekenhuisbacterie" MRSA). Om doelgericht deze ziekteverwekkers uit te schakelen is het van cruciaal belang om een gedegen kennis op te bouwen over het functioneren van de bacterie en hoe hij reageert op bedreigende condities (bv. hoge zoutconcentraties en hoge temperaturen). Kennis over factoren die bacteriële groei remmen, kan daarbij ook leiden tot de optimalisatie van de bereiding van producten waarbij van bacteriën gebruik wordt gemaakt (bv. kaas) of tot de ontwikkeling van zuivelproducten met bio-actieve cultures (bv. Yakult). In sommige kazen komen bijvoorbeeld hoge zoutconcentraties voor, en om melkzuurbacteriën onder deze condities optimaal te laten functioneren is het juist van belang de systemen die betrokken zijn bij de bescherming tegen zoutstress te stimuleren.

In dit proefschrift wordt bestudeerd hoe bacteriën reageren op een van de condities die voor hun bestaan bedreigend is, namelijk osmotische stress. Om een goed beeld te krijgen van de situatie kijken we eerst naar de opbouw van een bacteriële cel, wat osmotische stress is en wat de precieze effecten van osmotische stress zijn.

2. Hoe is een bacteriële cel opgebouwd?

De inhoud van een bacteriële cel, het zogenoemde cytoplasma (ofwel celvloeistof), bestaat uit een geconcentreerde oplossing van kleine (bv. suikers en aminozuren) en grote (bv.

eiwitten) organische moleculen en kleine geladen anorganische moleculen (ionen, ofwel geladen deeltjes). Ook het genetische materiaal (DNA) van de bacterie is gelokaliseerd in het cytoplasma. Het DNA levert de blauwdrukken (genen) voor de vele eiwitten die de cel kan synthetiseren. Deze eiwitten zijn te vergelijken met kleine machines/apparaten met elk een eigen functie die al naar gelang de behoefte van de cel op de juiste tijd en plaats gesynthetiseerd worden. Alle bovengenoemde moleculen spelen hun eigen rol in de processen die in de bacteriële cel plaatsvinden. Doordat de bacteriële cel is omgeven door een membraan, blijven deze moleculen in het cytoplasma gelokaliseerd (zie figuur 1). Deze cytoplasmatische membraan bestaat voornamelijk uit lipiden. Dit zijn vetmoleculen die zijn opgebouwd uit enerzijds een wateraantrekkende (hydrofiel) kopgroep en anderzijds een waterafstotend (hydrofoob) staartgedeelte. In een waterig milieu oriënteren de lipiden zich dusdanig dat er een continue membraan wordt gevormd die bestaat uit 2 laagjes lipiden (lipiden-bilaag). Hierbij groeperen de hydrofobe staarten van de lipiden zich in de zogenaamde 'hydrofobe kern' van de membraan, waarbij deze aan weerszijden door de hydrofiele kopgroepen wordt afgeschermd van water. De cytoplasmatische membraan functioneert als een fysieke barrière voor 'grote' moleculen, en de 'hydrofobe 'kern' van de cytoplasmatische membraan maakt dat het niet doorlaatbaar is voor geladen moleculen die wat betreft grootte wel de membraan zouden kunnen passeren. Het blijft echter doorlaatbaar voor water en kleine ongeladen moleculen Deze semi-doorlaatbare ('semi-permeabele') eigenschap van de membraan voorkomt echter ook dat voedingstoffen kunnen worden opgenomen en dat afvalstoffen kunnen worden uitgescheiden. Om dit toch mogelijk te maken bevinden zich in de membraan zogenoemde transporteiwitten (figuur 1). Deze overspannen de membraan en maken het mogelijk dat ook 'grote' en geladen moleculen de membraan kunnen passeren. Voor elk soort voeding- en/of afvalstof (zogenaamde substraten) bezit de cel



Figuur 1. Schematisch overzicht van de opbouw van een bacteriële cel.

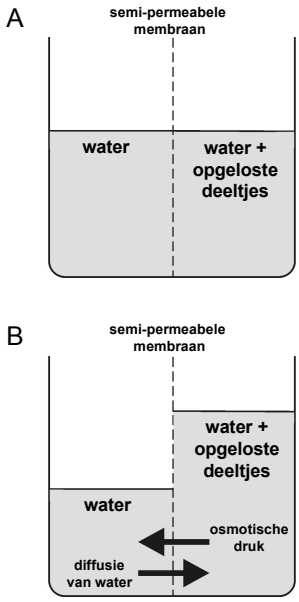
een apart transporteiwit, en afhankelijk van de aanwezigheid van een substraat wordt het betreffende transporteiwit aangemaakt door de cel. Deze selectieve opname stelt de cel in staat om, al naar gelang de behoefte, selectief bepaalde moleculen in de cel op te nemen (bv. voedingstoffen) of juist de cel uit te krijgen (bv. schadelijke stoffen). Op deze manier is het voor de cel ook mogelijk om een oneven verdeling van positief en negatief geladen deeltjes (ionen) aan weerszijden van de membraan te bewerkstelligen. Dit resulteert in een spanningsverschil over de membraan en kleine elektrische stroompjes die de cel voorzien van energie (te vergelijken met stroom uit het stopcontact). De cytoplasmatische membraan is vrij kwetsbaar en wordt daarom beschermd door een stug en sterk exoskelet, de zogenaamde celwand (figuur 1), en deze is doorlaatbaar voor zowel water als alle andere kleine moleculen. Kortom, de cytoplasmatische membraan zorgt ervoor dat het cytoplasma wordt afgeschermd van de omgeving terwijl de celwand vorm en stevigheid geeft aan de cel.

3. Wat is osmose?

De volgende term die verklaard moet worden is osmose. In figuur 2 wordt gedemonstreerd dat

wanneer twee compartimenten met enerzijds een waterige oplossing van een membraan-impermeabele stof (compartiment II) en anderzijds puur water (compartiment I) worden gescheiden door een semi-permeabele membraan er diffusie van water optreedt ten gevolge van een concentratiegradiënt van water (dit proces heet osmose). Deze gradiënt in de concentratie van water ontstaat doordat in de waterige oplossing deeltjes zijn opgelost die de concentratie van water verlagen ten opzichte van puur water. Zoals te zien is in figuur 2, vindt er geen netto diffusie van water meer plaats wanneer de druk, die wordt uitgeoefend door de extra hoeveelheid water in compartiment II, voor de diffusie van water compenseert. De druk die nodig is om de diffusie van water te compenseren wordt de osmotische druk van een oplossing genoemd. De osmotische druk die een waterige oplossing kan uitoefenen ten opzichte van puur water is afhankelijk van de concentratie van osmotisch actieve deeltjes, en deze wordt uitgedrukt in Osmol (de osmolaliteit van een oplossing).

Het cytoplasma van bacteriën is een geconcentreerde waterige oplossing en kan worden vergeleken met compartiment II uit het voorbeeld in figuur 2. Dat houdt in dat water vanuit de omgeving de cel in wil diffunderen. De hoeveelheid water die de cel in kan diffunderen is echter beperkt omdat de cytoplasmatische membraan beperkt elastisch is. Dit heeft tot gevolg dat er een naar buiten gerichte druk op de cytoplasmatische membraan komt te staan. Bij een te hoge druk zou dit leiden tot het knappen van de membraan (vergelijk met een ballon die te hard opgeblazen wordt), ware het niet dat een bacterie is omgeven door een sterke celwand. De celwand levert tegendruk aan de naar buiten gerichte druk, de zogenaamde turgor(-druk), waardoor er uiteindelijk geen netto diffusie van water meer plaatsvindt. De situatie is te vergelijken met wat er gebeurt tijdens het opblazen van een fietsband, waarbij de binnenband (cyto-plasmatische membraan) wordt opgeblazen en de buitenband (celwand) voorkomt dat de binnenband te ver uitzet. De opgebouwde druk



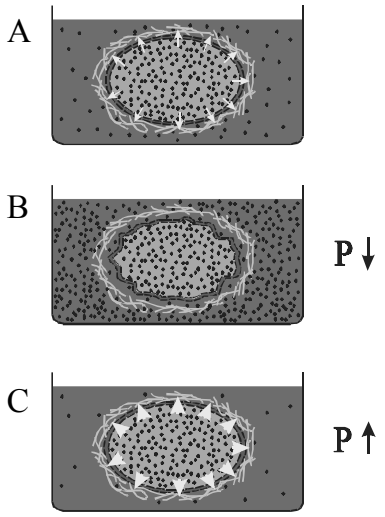
Figuur 2. De werking van osmose. Aan de start van het experiment is het systeem in osmotische onbalans (A). Na verloop van tijd belandt het systeem in een osmotisch evenwicht (B). De pijlen geven de beweging van water aan.

in de fietsband is te vergelijken met de turgor, en deze kan in bepaalde bacteriën oplopen tot ongeveer 20 atm. (dit is vergelijkbaar met 10 maal de druk in een fietsband!).

4. Wat is osmotische stress?

Een bacterie kan in zijn milieu geconfronteerd worden met twee typen osmotische stress (zie figuur 3). Ten eerste, een afname in de osmolaliteit van de omgeving (hypo-osmotische condities; figuur 3C). Door de resulterende diffusie van water de cel in wordt de turgor verhoogd, wat in extreme gevallen kan leiden tot het uiteenbarsten van de cel. In de cytoplasmatische membraan zitten echter eiwitten die gevoelig zijn voor het strekken/opspannen van de membraan, zogenaamde mechanosensitieve kanaaleiwitten. Deze kanaaleiwitten functioneren als overdrukventielen. Bij een te hoge druk openen deze eiwitten en vormen kanalen (poriën) in de cytoplasmatische membraan waardoor alle kleine moleculen die erdoor passen vanuit de cel in de omgeving worden geloosd. Dit voorkomt de ontwikkeling van een te hoge turgor en dus het knappen van de

membraan/celwand. Het tweede type van osmotische stress betreft een verhoging van de osmolaliteit van de omgeving (hyperosmotische condities; figuur 3B). De resulterende diffusie van water de cel uit leidt tot een afname van het celvolume en de turgor. Dit is te vergelijken met het leeglopen van een fietsband. Turgor is echter de drijvende kracht achter de groei van bacteriën en daarom is het van groot belang om het boven een bepaalde waarde te houden. Om dit te bewerkstelligen reageert een bacterie op een afname in de turgor door zogenoemde 'compatible solutes' in het cytoplasma te accumuleren. Dit leidt tot een verhoging van de osmolaliteit van het cytoplasma, diffusie van water de cel in, en het uiteindelijke herstel van turgor. Deze compatible solutes (zoals bv. glycine betaine) zijn moleculen die tot hoge concentraties in het cytoplasma kunnen worden geaccumuleerd zonder essentiële processen in de cel te verstoren. Ze zijn dus verenigbaar ('compatible') met de processen in de cel. Voor het overleven van de bacterie is het van het grootste belang dat deze compatible solutes zo snel mogelijk worden geaccumuleerd. Dit kan worden gerealiseerd doordat de bacterie deze stoffen opneemt uit de omgeving en/of ze zelf synthetiseert. Van deze twee opties is transport de meest directe en snelste oplossing, omdat het starten van de synthese van compatible solutes tijd kost. Uit eerder onderzoek is gebleken dat de transporteiwitten die zijn betrokken bij de opname van compatible solutes vaak gereguleerd worden door veranderingen in de osmolaliteit van de omgeving (osmotische regulatie). Dit houdt in dat wanneer de bacterie wordt onderworpen aan hyperosmotische condities ze meer van deze transporteiwitten gaan aanmaken en dat de activiteit van reeds bestaande transporteiwitten in de membraan wordt verhoogd (**hoofdstuk 2**). In tegenstelling tot de mechanosensitieve kanaaleiwitten ligt het mechanisme via welke deze transporteiwitten worden geactiveerd niet voor de hand. De doelstelling van het hier omschreven promotieonderzoek was het ophelderen van de wijze waarop hyperosmotische stress wordt gesignaleerd door de bacterie en via welk(e) mechanisme(n) vervolgens de transporteiwitten voor 'compatible solutes' worden geactiveerd.



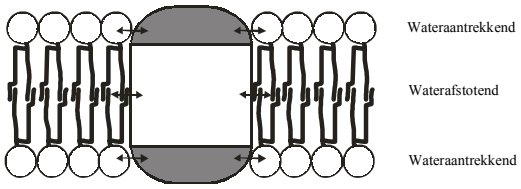
Figuur 3. Een bacterie is normalerwijze in balans met zijn omgeving (A). Zij kan echter worden blootgesteld aan hyperosmotische condities (B) of hypo-osmotische condities (C). De hoeveelheid stippen is een maat voor de concentratie van deeltjes. De pijlen zijn een weergave van de turgor (P).

5. Het feitelijke promotieonderzoek

Als modelorganisme werd in dit promotiewerk gebruik gemaakt van de melkzuurbacterie *Lactococcus lactis*, welke een grote rol speelt in de bereiding van zuivelproducten. Het transportsysteem dat in *L. lactis* betrokken is bij het transporteren van de compatible solute glycine betaine, het zogenoemde OpuA, is een ATP-binding cassette (ABC) transporter. Deze grote familie van transporters komt in bijna alle levende organismen voor en verbruikt ATP (adenosine-tri-fosfaat) om de opname van substraten te drijven. ATP is een hoogenergetische verbinding die vrij komt bij de afbraak van voedingsstoffen, en in alle levende organismen de grootste bron van metabolisch bruikbare energie is. In **hoofdstuk 5** is het beschreven hoe efficiënt OpuA omgaat met ATP. In vergelijking met andere bacteriële ABC transportersystemen, blijkt OpuA een afwijkende architectuur te bezitten (**hoofdstuk 6**). In deze samenvatting zal hier echter niet verder op worden ingegaan.

Voor het karakteriseren van de eigenschappen van een specifiek transportsysteem is het nodig om het te isoleren/scheiden van alle andere eiwitten in de cytoplasmatische membraan. Het geïsoleerde eiwit kan vervolgens worden teruggeplaatst in een kunstmatig membraan-systeem, resulterend in zogenoemde proteo-liposomen. De term kunstmatig slaat hier alleen op het feit dat de samenstelling van de lipiden in de membraan naar wens is te manipuleren. Deze proteoliposomen zijn te vergelijken met bacteriële cellen maar dan zonder cytoplasma en celwand. Hierdoor zijn ze in alle opzichten makkelijker manipuleerbaar en een stuk minder complex dan de originele cel. Door ATP (de energiebron voor transport) in te sluiten in deze proteoliposomen is het mogelijk de activiteit van OpuA te bepalen door de opname van radioactief gemerkte glycine betaine moleculen te volgen en/of door het verbruik van ATP te meten. Ook in dit kunstmatige modelsysteem bleek OpuA, net als in hele cellen, te kunnen worden geactiveerd door hyperosmotische condities (**hoofdstuk 3**). Een proteoliposoom, bestaande uit een membraan en OpuA, bezit dus alle eigenschappen om hyperosmotische condities waar te nemen en hierop te reageren. Omdat proteoliposomen geen celwand bezitten, en dus niet in staat zijn een turgor te verdragen (net als cellen zonder celwand; zie deel 4), is het uit te sluiten dat turgor een rol speelt in de activatie van OpuA. Gezien het feit dat er geen cytoplasma aanwezig is in proteoliposomen kon het ook worden uitgesloten dat er additionele eiwitten uit het cytoplasma nodig zijn voor de osmotische activatie van OpuA.

De volgende vraag die rijst is via welk mechanisme OpuA de hyperosmotische condities detecteert. Voelt OpuA de verandering in de osmotische condities direct (bv. verandering in de structuur van het transporteiwit onder rechtstreekse invloed van de osmotische condities) of indirect (bv. doordat OpuA, dat is gelokaliseerd in de membraan, veranderingen in de membraan detecteert ten gevolge van veranderende osmotische condities)? Membraaneiwitten overspannen de membraan en dit is mogelijk doordat het



Figuur 4. De waterafstotende (hydrofobe) en de wateraantrekkende (hydrofiele) delen van de membraan en de membraaneiwitten stemmen overeen.

centrale gedeelte van deze eiwitten, net als de membraan zelf (zie deel 2), hydrofoob (waterafstotend) is terwijl de boven- en onderzijde hydrofiel (wateraantrekkend) zijn (zie figuur 4). Doordat de lengte van het hydrofobe gedeelte van de eiwitten en de membraan overeenstemmen is het eiwit stabiel verankerd in de membraan. Transporteiwitten hebben dus een nauw contact met de lipiden in de membraan en zijn niet alleen gevoelig voor de dikte van de membraan maar ook voor de chemische eigenschappen van de lipiden. De kopgroep van een lipide kan neutraal of geladen zijn, de grootte van de kopgroep en de lengte van het staartgedeelte van een lipide kan variëren, etc.. Door systematisch deze parameters in de membraan van de proteoliposomen te variëren bleek dat de lading van de kopgroepen een grote rol speelt in de osmotische activatie van OpuA (**hoofdstuk 4**). Naarmate de fractie van negatief geladen lipiden in de membraan toeneemt is ook een hogere osmotische waarde nodig om OpuA te activeren. Tevens konden deze effecten worden nagebootst door gebruik te maken van kleine geladen moleculen die een specifieke interactie aangaan met de membraan. Deze geladen moleculen gaan tussen de kopgroepen van de lipiden zitten en verstoren op deze wijze de interacties van de ladingen (dit zijn zogenaamde elektrostatische interacties) tussen de kopgroepen en/of de interacties tussen de kopgroepen en OpuA. Samengenomen duiden de resultaten erop dat hyperosmotische stress de interacties tussen lipiden en/of lipiden en OpuA verstoort, resulterend in de 'indirecte' activatie van OpuA. Ook wordt uit deze resultaten duidelijk dat de bacterie de gevoeligheid van

OpuA voor hyperosmotische condities kan manipuleren door de samenstelling van de membraan te veranderen.

Hyperosmotische stress kan worden veroorzaakt door een toename in de concentratie van geladen deeltjes (bijvoorbeeld zouten) of ongeladen deeltjes (bijvoorbeeld suikers). In tegenstelling tot suikers staan zouten erom bekend dat ze de ladingsinteracties in de kopgroepregio van de membraan kunnen verstoren. Uit een zorgvuldige studie van de effecten van hyperosmotische condities ten gevolge van een toename in de concentratie van geladen of ongeladen moleculen bleek dat OpuA specifiek wordt geactiveerd door een verhoging van de concentratie van geladen moleculen aan de 'cytoplasmatische' zijde van OpuA. Het specifieke effect van geladen moleculen bevestigt dat ladingsinteracties tussen de lipiden en OpuA een belangrijke rol spelen in het mechanisme achter de osmotische activatie van OpuA.

Hoe kunnen deze resultaten worden vertaald naar wat er gebeurt in een bacterie als deze wordt geconfronteerd met hyperosmotische condities? Zoals beschreven is in deel 4, verliest een bacterie water en turgor ten gevolge van hyperosmotische condities. Door het verlies van water wordt het cytoplasma geconcentreerd en nemen de concentraties van zowel geladen (zoals zouten) als ongeladen (zoals suikers) moleculen toe. Het is echter bekend dat hoge concentraties van geladen deeltjes (zoals bv. zout-ionen) in het cytoplasma kunnen leiden tot de verstoring van processen die essentieel zijn voor het overleven van de cel. De verstoorde balans van geladen deeltjes (de zogenaamde elektrolyten-balans) in het cytoplasma heeft ook effecten op de lipiden- en/of lipiden-membraaneiwit interacties in de membraan. Dit leidt vervolgens tot de activatie van OpuA, de accumulatie van glycine betaine, diffusie van water de cel in, en het herstel van turgor en de elektrolyten balans.

6. Slotopmerkingen

Het werk dat wordt beschreven in dit proefschrift geeft meer inzicht in de manier waarop bacteriën omgaan met osmotische stress. De bevindingen zijn echter ook van toepassing op plant, mens en dier, omdat allerlei structuren in de cel (zoals bv. de cytoplasmatische membraan) zijn opgebouwd volgens universele principes. Zoals duidelijk blijkt uit dit werk spelen lipiden/membraaneiwit interacties een cruciale rol in het mechanisme achter de osmotische activatie van OpuA. De uitdaging voor de toekomst ligt dan ook in de identificatie van de delen van OpuA die de veranderingen in deze lipiden/eiwit interacties detecteren, en de lipiden/eiwit interacties die belangrijk zijn voor het functioneren van OpuA. Verder zal het interessant zijn om te bepalen of glycine betaine uitsluitend bescherming biedt tegen hyperosmotische stress. Het is namelijk bekend dat glycine betaine structuren in de cel (zoals eiwitten en de cytoplasmatische membraan) kan stabiliseren en het zou daarom de cel ook tegen andere soorten stress kunnen beschermen. Mocht dit zo zijn dan kunnen transportsystemen zoals OpuA een sleutelpositie innemen in de strijd tegen bacteriële infecties. De verdere ontrafeling van hoe bacteriën weerstand bieden aan stress (bv. osmotische stress, temperatuurstress en stress ten gevolge van antibiotica) is dan ook van groot belang om strategieën te ontwikkelen waarmee we bacteriën in toom kunnen houden.

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